Chemosensitivity of Lymphocytes From Patients With B-Cell Chronic Lymphocytic Leukemia to Chlorambucil, Fludarabine, and Camptothecin Analogs

By Robert Silber, Barbara Degar, Dan Costin, Elizabeth W. Newcomb, Mathew Mani, Carl R. Rosenberg, Laura Morse, John C. Drygas, Zoe N. Canellakis, and Milan Potmesil

Chemosensitivity of B lymphocytes, obtained from 65 patients with B-cell chronic lymphocytic leukemia (B-CLL), was determined using the MTT assay. The results were expressed by the drug concentration required for 50% inhibition of cell viability (IC50). The cytotoxicity of chlorambucil (CLB) was compared with that of fludarabine and the DNA topoisomerase I inhibitors, camptothecin, 9-aminocamptothecin, 10,11-methylenedioxy-20(S)-camptothecin (10,11-MDC), and 9-amino-10,11-methylenedioxy-20(S)-camptothecin (9-A-10,11-MDC), and topotecan. Considerable heterogeneity in sensitivity to CLB was observed, with a median IC50 of 40.5 μmol/L in untreated patients. B-CLL cells from patients treated with CLB had a significantly higher median IC50 of 86.0 μmol/L (P < .01). Untreated as well as CLB-treated patients were divided into two subsets. For the purpose of this study, B-CLL lymphocytes with an IC50 CLB of less than 61.0 μmol/L were designated as "sensitive," and those with an IC50 CLB of ≥61.0 μmol/L were designated as "resistant." After baseline assays, 15 untreated patients received CLB; after treatment, the IC50 increased in B-CLL lymphocytes from 13 of 15 patients. The response to CLB treatment, determined by its effect on the absolute lymphocyte count and by the Eastern Cooperative Oncology Group clinical criteria, was significantly better in patients whose lymphocytes had an IC50 CLB of less than 61.0 μmol/L before therapy (P < .01). B-CLL lymphocytes also had a variable degree of sensitivity in vitro to each of the other drugs. There was significant cross-resistance between CLB and fludarabine (P < .001). Whereas only 29% of CLB-resistant B-lymphocyte specimens obtained from individual patients were sensitive to fludarabine in vitro, 52% and 67% of CLB-resistant lymphocyte samples were sensitive to 10,11-MDC and 9-A-10,11-MDC, respectively. We have previously reported that p53 gene mutations were associated with aggressive B-CLL and a poor prognosis. B lymphocytes from seven patients with these mutations were resistant to CLB, and five of six were resistant to fludarabine. Lymphocytes from four of seven were resistant to 10,11-MDC, and three of four were resistant to 9-A-10,11-MDC. This study implies that the MTT assay may be useful in identifying subsets of CLL patients resistant to conventional chemotherapy. However, definitive conclusions cannot be drawn in view of the small number of patients studied prospectively. In addition, these results suggest the potential of camptothecin-based therapy for patients unresponsive to standard treatment.

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Chronic Lymphocytic Leukemia (CLL) is an incurable clonal disease with a variable response to therapy. Until recently, standard treatment had been chlorambucil (CLB) with or without corticosteroids. Over the last decade, three nucleoside analogs, pentostatin, fludarabine, and 2′chlorodeoxyadenosine, have become available for treatment of CLL. Among these, fludarabine has been most widely used. Among these, fludarabine has been most widely used.

An in vitro assay predicting the clinical response to a particular agent would be of value in the selection of drugs with which to treat CLL. To this end, several studies have determined chemosensitivity using a dye exclusion method (such as cytosine arabinoside), or with an MTT assay. These earlier studies evaluated the efficacy of CLB, as compared with other drugs, and its synergism with interferon. Using the MTT assay, in this report, we present longitudinal studies with patients whose lymphocytes showed varying degrees of sensitivity to CLB. Our results show a concordance between the in vitro response to CLB and the effect of therapy on the patient’s clinical and absolute lymphocyte count (ALC) response.

In the present study, we also provide data that compare the cytotoxicity of fludarabine, camptothecin (CPT), and several of its analogs with that of CLB in CLL B lymphocytes. The CPTs are inhibitors of DNA topoisomerase I. Among these drugs, 9-aminocamptothecin (9-A-CPT) and 10,11-methylenedioxy-20(S)-camptothecin (10,11-MDC) showed an unprecedented effectiveness against various resistant human cancers carried as xenografts by immunodeficient mice. In phase II studies, the analog 7-ethyl-10-[4-((1-piperidyl)-1-piperidyl)-carbonyloxycamptothecin showed substantial activity against a variety of carcinomas, as well as against non-Hodgkin’s lymphoma and acute leukemia. Several CPTs are undergoing preclinical screening, and some are already in clinical trials. We now show that 10,11-MDC and 9-amino-10,11-methylenedioxy-20(S)-camptothecin (9-A-10,11-MDC) are effective in killing B-cell CLL (B-CLL) B lymphocytes in vitro.

We have previously documented that the presence of mutations in the p53 gene had an adverse effect on the prognosis of CLL: the present study reports the correlation between these mutations and chemosensitivity to fludarabine, 10,11-MDC, and 9-A-10,11-MDC.
Our results indicate that the MTT assay may be a valid predictor of the clinical response to CLB. In addition, the CPTs were found to be more cytotoxic than fludarabine to CLB-resistant B-CLL lymphocytes in vitro.

MATERIALS AND METHODS

Patient population. A total of 65 patients with B-CLL seen at the New York University Medical Center (New York, NY) were studied. The diagnosis of B-CLL required the demonstration of at least 5 X 10^9/L monoclonal B lymphocytes positive for CD5, CD19, and CD20. The disease was staged according to Rai et al.22 The patient distribution was as follows: stage 0, 24; stage I, 17; stage II, 5; stage III, and 1 stage IV. The patients were divided into untreated and treated groups. The untreated group consisted of 43 patients who had never received treatment and 3 patients who had been treated more than 1 year before this study. The treated group consisted of 34 patients, 15 of whom were originally included in the untreated patient group. In addition to earlier CLB treatment with or without prednisone, 9 patients had received cyclophosphamide; 2, fludarabine; 2, etoposide or pentostatin; and single patients received either vincristine, mitoxantrone, or CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone). Responses were evaluated according to the criteria of the EST 2480 Eastern Cooperative Oncology Group (ECOG) protocol.23

Lymphocyte isolation. Heparinized blood was obtained from consenting patients. Mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradients (PH Pharmacia, Uppsala, Sweden); T lymphocytes were removed by sheep erythrocyte rosette formation, and monocytes were depleted by adherence to Falcon plastic dishes (Fisher, Springfield, NJ). Enrichment of B lymphocytes was assessed by flow cytometry with anti-CD5, anti-CD19, and anti-CD20 antisera. Purity of greater than 95% B-CLL B lymphocytes with anti-CD3, was obtained.

Tissue culture. A T-cell acute lymphoblastic leukemia line RPMI 8402 and its CPT-K5 subline, which is resistant to CPT-11 (Research Triangle Institute, Research Triangle Park, NC). Stock solutions of the CPTs in DMSO (1.0 to 6.0 mmol/L) were established spectrophotometrically. The follow-

RESULTS

Correlation of MTT assay results with response to CLB treatment. The drug concentration required for 50% inhibition of cell viability (IC_{50}) of CLB in B lymphocytes from untreated patients ranged from 7 to 275 μmol/L. The median IC_{50} in this group (Fig 1A) was 40.5 μmol/L (n = 46), whereas cells from patients who were treated with CLB or with CLB and prednisone (Fig 1B) within 12 months before the MTT assay had a higher median IC_{50} value of 86.0 μmol/L (n = 34; P < .01). In both the treated and untreated groups, the preparation used in the MTT assay contained more than 90% CD5+ B lymphocytes. No correlation was found between IC_{50} and Rai stage.

The median IC_{50} CLB for the total (treated and untreated) patient group was 61.0 μmol/L (n = 80; see Fig 1 [- - - - -]). A nonparametric test showed no evidence against randomness either in the less than 61.0 μmol/L or the ≥61.0 μmol/L subset. However, this test of serial randomness was significantly positive for the pooled data (P < .005), suggesting
no. 2, 3, cytotoxicity had an IC₅₀ less than 61.0 pmol/L during baseline. With no response, 1 had a partial remission, and 2.

The difference in partial remissions between the two groups is also significant by Fisher's exact test.

The chemosensitivity of lymphocytes from treated and untreated patients to fludarabine, 10,11-MDC, and 9-A-10,11-MDC is shown (Fig 4). The selected limits of sensitivity for each drug (Fig 4 [---]) were defined as the mean IC₅₀ plus 2 SD. As with CLB, lymphocytes with an IC₅₀ value above these limits were defined as resistant. The IC₅₀ values for each drug are shown for cells sensitive to CLB (IC₅₀ < 61.0 μmol/L) and CLB resistant (IC₅₀ ≥ 61.0 μmol/L). The IC₅₀ for fludarabine ranged from 6 to greater than 100 μmol/L; the IC₅₀ for CLB-sensitive was lower than that of the CLB-resistant group (Fig 4A; P < .01). As for CLB, a nongenetic test of serial randomness applied to pooled data was significantly positive (P < .01) suggesting the presence of two runs with a clustering of lower and higher IC₅₀s.

For 10,11-MDC (IC₅₀, 0.10 to greater than 1.0 μmol/L) and 9-A-10,11-MDC(IC₅₀, 0.03 to greater than 1.0 μmol/L), there were no significant differences (P > .05) between the subsets of CLB-sensitive and CLB-resistant B lymphocytes. In an earlier study, we reported the relationship between CLB resistance and p53 gene mutations. Lymphocytes with these mutations were also observed with fludarabine in cells from 23 of 33 patients, as well as with 10,11-MDC in 17 of 24 patients, and with 9-A-10,11-MDC in 22 of 23 patients. CLB-resistant lymphocytes from 13 of 23 patients were sensitive to 10,11-MDC, and 10 of 15 were sensitive to 9-A-10,11-MDC. In contrast only 7 of 24 CLB-resistant lymphocyte preparations were sensitive to fludarabine.

When compared with 10,11-MDC and 9-A10, 11-MDC, equitoxic concentrations were generally higher for CPT (N = 5), 9-AC (N = 19), and topotecan (N = 11), with median IC₅₀ values of approximately 1.0 μmol/L, 1.0 μmol/L, and 1.6 μmol/L, respectively. There was no difference in median IC₅₀ values for B-CLL lymphocytes between treated and untreated patients.

CPT-sensitive and CPT-resistant tissue-culture lines were used as controls for drug sensitivity. The parent RPMI 8402 line was sensitive to 10,11-MDC and 9-A-10,11-MDC (Fig 4), whereas the CPT-K5 subline showed resistance to these drugs.

Relationship of drug resistance to p53 mutations. In an earlier study, we reported the relationship between CLB resistance and p53 gene mutations in B-CLL lymphocytes. Chemosensitivity of B lymphocytes to fludarabine and the CPTs was compared with that of CLB and correlated with the presence of p53 mutations. Lymphocytes with these mutations from all 7 patients were resistant to CLB, and 5 of 6 were resistant to fludarabine (Table 2). Lymphocytes from
Table 1. Effect of Therapy on Response

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Rai Stage</th>
<th>Therapy</th>
<th>ECOG Clinical Response</th>
<th>ALC (x10^3/µl) Pretreatment</th>
<th>% Decrease in ALC</th>
<th>Chlorambucil IC₅₀ Avg Pretreatment</th>
<th>IC₅₀ Ratio Post/Pre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>III</td>
<td>CLB</td>
<td>PR</td>
<td>497</td>
<td>93</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>CLB</td>
<td>PR</td>
<td>63</td>
<td>87</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>II</td>
<td>CLB + PRED</td>
<td>PR</td>
<td>44</td>
<td>77</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>II</td>
<td>CLB</td>
<td>PR</td>
<td>76</td>
<td>80</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>I</td>
<td>CLB</td>
<td>PR</td>
<td>82</td>
<td>93</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>II</td>
<td>CLB</td>
<td>PR</td>
<td>133</td>
<td>92</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>II</td>
<td>CLB + PRED</td>
<td>PR</td>
<td>285</td>
<td>84</td>
<td>28</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>II</td>
<td>CLB + PRED</td>
<td>PR</td>
<td>115</td>
<td>73</td>
<td>34</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>II</td>
<td>CLB</td>
<td>PR</td>
<td>82</td>
<td>74</td>
<td>38</td>
<td>&gt;9</td>
</tr>
<tr>
<td>10</td>
<td>I</td>
<td>CLB</td>
<td>PR</td>
<td>41</td>
<td>90</td>
<td>41</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>I</td>
<td>CLB + PRED</td>
<td>PR</td>
<td>55</td>
<td>82</td>
<td>54</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>II</td>
<td>CLB</td>
<td>PR</td>
<td>41</td>
<td>86</td>
<td>66</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>I</td>
<td>CLB + PRED</td>
<td>DP</td>
<td>262</td>
<td>0</td>
<td>103</td>
<td>2</td>
</tr>
<tr>
<td>14</td>
<td>III</td>
<td>CLB</td>
<td>DP</td>
<td>25</td>
<td>0</td>
<td>112</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>II</td>
<td>CLB + PRED</td>
<td>NR</td>
<td>190</td>
<td>49</td>
<td>124</td>
<td>2</td>
</tr>
</tbody>
</table>

Abbreviations: PRED, prednisone; PR, partial response; NR, no response; DP, disease progression; ECOG, Eastern Cooperative Oncology Group.

4 of 7 patients were resistant to 10,11-MDC, and 3 of 4 were resistant to 9-A-10,11-MDC.

Chemosensitivity of B lymphocytes without p53 mutations from 39 patients was also analyzed in terms of CLB resistance. Among the 19 patients with CLB-sensitive lymphocytes, 15 of 17 tested were also sensitive to fludarabine, 14 of 17 were sensitive to 10,11-MDC, and 15 of 15 were sensitive to 9-A-10,11-MDC. Among the 20 patients with CLB-resistant lymphocytes, 6 of 18 tested were sensitive to fludarabine, 9 of 16 tested were sensitive to 10,11-MDC, and 6 of 6 tested were sensitive to 9-A-10,11-MDC.

Fig 3. Effect of therapy on IC₅₀ CLB and ALC in 6 representative patients. Patients no. 2, 3, 5, and 7 from Table 1 had B lymphocytes with pretreatment IC₅₀ CLB values in the sensitive range, and patients no. 13 and 15 had pretreatment IC₅₀ CLB values in the resistant range. Patients no. 2, 3, and 5 were treated with CLB; patients no. 7, 13, and 15 (1-1) were treated with CLB and prednisone. Patient no. 13 was also treated with fludarabine (1-1).
to 10,11-MDC, and 9 of 11 were sensitive to 9-A-10,11-MDC.

**DISCUSSION**

The MTT assay has been used to predict the response to therapy\(^2\) and to investigate drug resistance in relapsed acute leukemia.\(^2\) The assay has been less widely applied to the lymphoid malignancies, including CLL.\(^2\) In this report, we establish a positive correlation between in vitro chemosensitivity to CLB and a favorable response to treatment in B-CLL. We have also investigated the in vitro cross-resistance of CLB to fludarabine as well as to CPT and its analogs, a new class of anticancer agents. Three findings with CLB emerge from this study. The first is the marked heterogeneity in the in vitro sensitivity to CLB and the other drugs investigated.

Cells from the majority of untreated patients are sensitive to CLB, whereas cells from about one third of the cases are consistently more resistant to this drug. This may reflect a manifestation of de novo resistance, a common and serious problem in chemotherapy. The second is the positive correlation between the in vitro chemosensitivity of CLL B lymphocytes and the patients' response to CLB treatment. The well-recognized variable therapeutic efficacy of CLB may be the in vivo counterpart of the heterogeneity in cytotoxicity observed in vitro. Further studies with a larger number of patients will be required to determine if the MTT assay has a predictive value in the treatment of B-CLL.

A third finding documents the effect of therapy on the CLB resistance observed in vitro after treatment occurred in the leukemic CD5+ B lymphocytes. The increase in IC\(_{50}\) CLB was not caused by the presence of T cells or monocytes, because T-cell depletion was complete and monocyte contamination was below 1%. Although some of the 15 patients studied prospectively received prednisone as well as CLB, the effect of therapy on IC\(_{50}\) was the same in patients

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**Table 2. Relationship of Chemosensitivity to Lymphocyte p53 Mutations**

<table>
<thead>
<tr>
<th>p53 Mutations</th>
<th>Fludarabine</th>
<th>10,11-MDC</th>
<th>9-A-10,11-MDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLB-resistant</td>
<td>5</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not found</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLB-resistant</td>
<td>12</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>(n = 20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLB-sensitive</td>
<td>2</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>(n = 19)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p53 Mutations were detected as previously described.\(^5\) Chemosensitivity of B lymphocytes was assayed as described under Materials and Methods. Data in table refer to number of patients who were studied.
receiving CLB alone as in those who were also treated with prednisone. The increase in IC50 most likely stems from the killing of sensitive lymphocytes by CLB, with selection of a resistant subpopulation. The increase observed in IC50 in some cases appears reversible, despite continued therapy. The fluctuations in IC50 observed in about one fifth of the untreated patients indicate that factors other than CLB therapy can cause changes in drug resistance that are reflected in the IC50. We think it is not an artifact of the assay. The nature of these factors remains unclear.

In the second part of the study, the in vitro effects of fludarabine and the CPTs were evaluated. The results with fludarabine showed considerable cross-resistance with CLB. A clinical response to fludarabine in CLB-resistant patients is amply documented. In contrast, our findings of cross-resistance in vitro is consistent with the clinical observation that fludarabine does not prolong the survival of patients resistant to CLB (and prednisone). Our in vitro results differ from those of others who, using another chemosensitivity assay, find no concordance in cross-resistance between CLB and fludarabine.

We have previously reported the association between p53 gene mutations and CLB resistance in CLL lymphocytes. The present work shows that resistance to fludarabine and the CPTs also occurs commonly in lymphocytes with a mutated p53 gene.

Our study also reports the effects of CPT and several of its analogs on CLL lymphocytes. Among these agents, all topoisomerase I inhibitors, two analogs, 10,11-MDC and 9-A-10,11-MDC, showed the greatest activity against CLL lymphocytes. The first of these agents introduces a greater number of breaks into CLL lymphocyte DNA than do CPT or 9-AC. In addition, when CLL lymphocytes are incubated with 10,11-MDC, the amount of cell-associated drug is higher and its retention in the lactone form is greater than that observed with CPT or 9-AC. These differences may not be solely responsible for the greater cytotoxicity of 10,11-MDC and 9-A-10,11-MDC. Although it is tempting to suggest that the greater cytotoxicity is caused by the increased number of breaks induced by these compounds in CLL lymphocytes, the underlying mechanisms for cell killing by these agents remains not completely understood.

The extreme S-phase cytotoxicity of CPT has been extensively documented. In the “collision” model, the interaction between the advancing DNA replication fork and the topoisomerase I-CPT-cleaveable complex results in a double-strand DNA break. This event, which triggers S-phase-specific cell killing and G2-phase cell cycle arrest, may be responsible for the highly selective killing of the S-phase cells; however, it cannot solely account for the impressive cytotoxicity to quiescent B-CLL lymphocytes, which are largely arrested at the G0 to G1 phase of the cell cycle. Additional processes may be involved.

CLB is an alkylating agent. Fludarabine, a DNA elongation terminator, is a DNA ligase inhibitor. The CPT analogs, 10,11-MDC and 9-A-10,11 MDC, are DNA topoisomerase I inhibitors. The reason for the occurrence of cross-resistance to these agents that have different mechanisms of action remains unknown. A common explanation may reside in the cell’s ability to repair DNA damage caused by chemotherapeutic agents. The increased expression in B-CLL lymphocytes of the ERCC-1 gene, which is involved in radiation damage repair, supports such an interpretation. Another mechanism may involve the induction of heat-shock proteins recently described in CLL lymphocytes. These proteins that are induced by several cytostatic drugs may be associated with drug resistance.

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