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

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.

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 to measure cell viability. More recently, a viability assay has been tested in CLL. Results with the MTT assay correlated well with the dye exclusion method. 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 1. Among these drugs, 9-aminocamptothecin (9-AC) 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)-carboxyloxy]camptothecin 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 $5 \times 10^9$UL monoclonal B lymphocytes positive for CD5, CD19, and CD20. The disease was staged according to Rai et al. The patient distribution was as follows: 18 patients, 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; 5, 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. Lympocyte 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 fewer than 2% T lymphocytes, as determined by flow cytometry with anti-CD3, was obtained by this procedure.

Tissue culture. A T-cell acute lymphoblastic leukemia line RPMI 8402 and its CPT-K5 subline, which is resistant to CPT-11, were obtained from Dr. T. Andoh (Aichi Cancer Center Research Institute, Nagoya, Japan) and grown as a cell suspension in RPMI 1640-10% fetal calf serum in the presence of drugs or drug combinations. RPMI 8402 and its CPT-K5 subline, which is resistant to CPT-11, was assessed by Fisher's exact test for nominal variables and by a nonparametric test of serial randomness. Values in all analyses were two-tailed. The SAS system was used for all statistical calculations.

RESULTS

Correlation of MTT assay results with response to CLB treatment. The drug concentration required for 50% inhibition of cell viability (IC50) of CLB in B lymphocytes from untreated patients ranged from 7 to 275 μmol/L. The median IC50 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 IC50 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 IC50 and Rai stage.

The median IC50 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...
the presence of two runs (clumps), one at the low and the other at the high range of IC\textsubscript{50} values. Based on this analysis, B lymphocytes with IC\textsubscript{50} CLB less than 61.0 \(\mu\text{mol/L} \) were designated as ‘sensitive,’ and those with IC\textsubscript{50} CLB \(\geq 61.0 \mu\text{mol/L} \) were designated as ‘resistant’ within the context of this study.

Fifteen patients were entered into a prospective study to correlate the pretreatment IC\textsubscript{50} CLB with the decrease in ALC and clinical response. The baseline pretherapy and maximal posttherapy IC\textsubscript{50} values for 15 previously untreated patients were determined (Fig 2). The median posttherapy IC\textsubscript{50} was 5 times the median baseline pretreatment value, with an increase in IC\textsubscript{50} of up to 20-fold after therapy. The increase was observed as early as 2 weeks and as late as 3 months after therapy was instituted. With a maximal follow-up time of 2 years, the IC\textsubscript{50} remained elevated in 9 of 13 surviving patients. Among the 46 untreated patients on whom serial assays were performed over a 2-year period, we noted 10 instances of fluctuations in IC\textsubscript{50} (data not shown). The effect of therapy on the clinical response and ALC is shown in Table 1. Eleven patients whose lymphocytes had an IC\textsubscript{50} less than 61.0 \(\mu\text{mol/L} \) during baseline studies all had a partial clinical remission and showed a greater than 70% decrease in ALC after therapy. In contrast, of the 4 patients whose lymphocytes had an IC\textsubscript{50} \(\geq 61.0 \mu\text{mol/L} \), 1 had no response, 1 had a partial remission, and 2 had disease progression. The difference in partial remissions between the two groups is also significant by Fisher’s exact test.

During treatment, there was a rapid increase in IC\textsubscript{50} concomitant with a decrease in ALC. The change was noted as early as within 1 week (patient no. 2) and as late as 6 months (patient no. 5). Beyond the observation of an increase in IC\textsubscript{50}, the small number of patients hampers further correlation between therapy and IC\textsubscript{50} oscillations. Lymphocytes from patients no. 13 and 15 had pretreatment IC\textsubscript{50} CLB values that placed them in the resistant group. With therapy these also showed an increase in IC\textsubscript{50} and a decrease in ALC, but the ALC remained greater than 60 \(\times 10^3/\mu\text{L} \).

Comparison of CLB, fludarabine, and the camptothecins. 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 IC\textsubscript{50} plus 2 SD. As with CLB, lymphocytes with an IC\textsubscript{50} value above these limits were defined as resistant. The IC\textsubscript{50} values for each drug are shown for cells sensitive to CLB (IC\textsubscript{50} < 61.0 \(\mu\text{mol/L} \) and CLB resistant (IC\textsubscript{50} \(\geq 61.0 \mu\text{mol/L} \)). The IC\textsubscript{50} for fludarabine ranged from 6 to greater than 100 \(\mu\text{mol/L} \); the IC\textsubscript{50} for CLB-sensitive was lower than that of the CLB-resistant group (Fig 4A; \(P < .01 \)). As for CLB, a nonparametric 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 IC\textsubscript{50}.

For 10,11-MDC (IC\textsubscript{50} 0.10 to greater than 1.0 \(\mu\text{mol/L} \)) and 9-A-10,11-MDC(IC\textsubscript{50} 0.03 to greater than 1.0 \(\mu\text{mol/L} \)), there were no significant differences (\(P > .05 \)) between the subsets of CLB-sensitive and CLB-resistant B lymphocytes (Fig 4B and C), and there was no evidence against randomness of IC\textsubscript{50} distribution. Among the CLB-sensitive cells, there is a cluster with low IC\textsubscript{50} values. Clusters 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, equivalent concentrations were generally higher for CPT (\(N = 5 \), 9-AC (\(N = 19 \)), and topotecan (\(N = 11 \)), with median IC\textsubscript{50} values of approximately 1.0 \(\mu\text{mol/L} \), 1.0 \(\mu\text{mol/L} \), and 1.6 \(\mu\text{mol/L} \), respectively. There was no difference in median IC\textsubscript{50} 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.\textsuperscript{11} 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
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

<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>Maximal % Decrease in ALC</th>
<th>Chlorambucil</th>
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<tr>
<td>1</td>
<td>III</td>
<td>CLB</td>
<td>PR</td>
<td>497</td>
<td>93</td>
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<td>PR</td>
<td>63</td>
<td>87</td>
<td>11</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>
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<td>5</td>
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<td>PR</td>
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<td>93</td>
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<td>II</td>
<td>CLB</td>
<td>PR</td>
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<td>CLB + PRED</td>
<td>PR</td>
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<td>84</td>
<td>28</td>
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<td>8</td>
<td>II</td>
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<td>PR</td>
<td>115</td>
<td>73</td>
<td>34</td>
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<tr>
<td>9</td>
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<td>PR</td>
<td>82</td>
<td>74</td>
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<tr>
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<td>PR</td>
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<td>90</td>
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<td>11</td>
<td>I</td>
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<td>55</td>
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<td>13</td>
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<td>CLB</td>
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<td>15</td>
<td>II</td>
<td>CLB + PRED</td>
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<td>49</td>
<td>124</td>
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</tbody>
</table>

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

Fig 3. Effect of therapy on IC_{50} CLB and ALC in 6 representative patients. Patients no. 2, 3, 5, and 7 from Table 1 had B lymphocytes with pretreatment IC_{50} CLB values in the sensitive range, and patients no. 13 and 15 had pretreatment IC_{50} CLB values in the resistant range. Patients no. 2, 3, and 5 were treated with CLB; patients no. 7, 13, and 15 (I-) were treated with CLB and prednisone. Patient no. 13 was also treated with fludarabine (I-).
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 and to investigate drug resistance in relapsed acute leukemia. The assay has been less widely applied to the lymphoid malignancies, including CLL. 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 IC₅₀ CLB. When cells from treated patients were compared with cells from patients who had not received CLB, the median IC₅₀ CLB of the former group was significantly higher than the median IC₅₀ of the latter. The possibility was considered that the higher IC₅₀ in patients needing treatment merely reflected a more advanced disease stage rather than a consequence of CLB therapy. Contrary to this concept is the lack of correlation between Rai stage and IC₅₀. Further evidence against this interpretation is the relatively rapid increase in IC₅₀ CLB observed after therapy that was not noted in untreated patients.

The CLB resistance observed in vitro after treatment occurred in the leukemic CD5+ B lymphocytes. The increase in IC₅₀ 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 IC₅₀ was the same in patients.

**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>
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</thead>
<tbody>
<tr>
<td>Present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLB-resistant</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Not found</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 1)</td>
</tr>
<tr>
<td>CLB-resistant</td>
<td>12</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>(n = 20)</td>
<td>12</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>CLB-sensitive</td>
<td>2</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>(n = 19)</td>
<td>2</td>
<td>15</td>
<td>3</td>
</tr>
</tbody>
</table>

p53 Mutations were detected as previously described. 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.6

We have previously reported the association between p53 gene mutations and CLB resistance in CLL lymphocytes.21 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.32 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.19,33 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-cleavable complex results in a double-strand DNA break.34 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.34

CLB is an alkylating agent. Fludarabine, a DNA elongation terminator, is a DNA ligase inhibitor.25 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.37 These proteins that are induced by several cytostatic drugs may be associated with drug resistance.24,39

REFERENCES


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