Loxoribine Induces Chronic Lymphocytic Leukemia B Cells to Traverse the Cell Cycle

By Michael G. Goodman, Susan B. Wormsley, John C. Spinosa, and Lawrence D. Piro

Leukemic B cells from a majority of patients with chronic lymphocytic leukemia (CLL) enter the cell cycle upon stimulation in vitro with loxoribine, a potent 7,8-disubstituted guanine ribonucleoside immunostimulant. In the absence of added costimulants, a proportion of these cells become activated and undergo DNA synthesis and mitosis accompanied by a marked increase in expression of an array of cell surface activation antigens. The resultant activated B-CLL cells exhibit greatly enhanced sensitivity to cycle-active cytotoxic drugs. This approach may be of potential value in the therapy of CLL.

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Loxoribine was synthesized and generously provided by Dr Allen Reitz (R.W.J. Pharmaceutical Research Institute, Spring House, PA).

IN ITS EARLY STAGES, chronic lymphocytic leukemia (CLL) is an indolent disease, one in which malignant cells accumulate through lengthened life span rather than accelerated proliferation. This may be largely caused by increased expression of the bcl-2 gene product or by production of interferon γ (IFNγ) by the malignant B cells and resultant inhibition of apoptosis. In the later stages of CLL, which are characterized by significant anemia and/or thrombocytopenia, prognosis worsens and median survival is less than two years. This disease is difficult to treat effectively because of the very low rate of cellular proliferation, and cures are virtually unknown. The idea was proposed long ago that if CLL cells could be induced to cycle transiently, ie, without undergoing permanent transformation, the disease would be amenable to treatment with intent to cure by using cycle-active cytotoxic drugs. A number of agents have been tested in this regard, with varying degrees of success in vitro: bacterial endotoxin, anti-μ antibodies with and without phytohemagglutinin (PHA)-conditioned medium, Staphylococcus aureus protein A from Cowan I (SAC), thiorodoxin, phorbol esters, and a number of cytokines individually and in combination. Endotoxin and phorbol esters per se cannot be used in humans. Anti-μ antibodies would likely affect normal as well as malignant B cells; this is complicated further because B-CLL cells express sIg at very low density. Stimulation by cytokines appears to require an initiation signal such as anti-μ antibodies, SAC, or 12-0-tetradecanoylphorbol-13-acetate (TPA).

Loxoribine and its analogues are well-characterized pleiotropic agonists of immune responses in a variety of species, including man. The present studies were undertaken to investigate the capacity of loxoribine to induce cell-cycle entry by B-CLL cells, and the effects of any such action on cellular sensitivity to cycle-active cytotoxic drugs.

MATERIALS AND METHODS

Cell preparation. Human peripheral blood mononuclear cells (PBMC) from patients with CLL or hairy cell leukemia (HCL) were prepared from heparinized or citrated venous blood by ficoll-diatrizoate density gradient centrifugation. Viability, determined by trypan blue dye exclusion, was ≥95%.

Lymphocyte culture. The tissue-culture medium used in these experiments, containing 10% heat-inactivated autologous plasma in an RPMI-1640-based medium was prepared as described previously but without interleukin-2 (IL-2) supplementation. B-CLL cells enjoyed prolonged viability in this medium. In our experience, use of fetal calf serum was significantly inhibitory. Cultures were incubated for 72 hours unless otherwise indicated.

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Cell proliferation assay. PBMC from leukemic subjects were incubated in 96-well microculture plates. One hundred microliters of PBMC at 10^6/mL was plated in replicates of five and incubated with culture medium as a control or with incremental quantities of loxoribine as indicated. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Cultures were pulsed with 0.6 μCi [3H]Tdr (ICN Pharmaceuticals, Costa Mesa, CA) for the final 24 hours of the 72-hour culture period. Cells were harvested on a multi-sample automated harvester, [3H]Tdr uptake was measured by liquid scintillation spectrometry.

Histopathology and immunohistochemistry. PBMCs from leukemia patients were cultured with medium (control) or with loxoribine as described above. After 2 to 3 days' incubation, 50 μL of cultured cell suspension was transferred to microscopic slides with a cytocentrifuge. Slides were then stained with Wright-Giemsa stain according to manufacturer's recommendation (Hema-Tek, Miles Inc, Elkhart, IN).

Immunohistochemistry using antibody against κ-light chain was performed on cytocentrifuged microscopic slides prepared with 50 μL of cultured cell suspension. The prepared slides were fixed in cold acetone and incubated with rabbit polyclonal antibody against κ-light chain as described. Detection of primary antibody was accomplished with horseradish peroxidase-conjugated antirabbit Ig and DAB as substrate. Tartrate inhibition of acid phosphatase activity was assessed in cytocentrifuged preparations using commercially available test kits (Sigma Chemical Co, St Louis, MO), without modification of the manufacturer's instructions.

Immunophenotyping. PBMC from leukemic patients were cultured with medium (control) or with incremental concentrations of loxoribine as shown. Thirty-six replicate wells for each culture condition were plated. After incubation, pooled cells from triplicate wells were incubated with 150 μL of antibody cocktail (Table 1) for 30 minutes, washed twice in phosphate-buffered saline (PBS) containing 2% heat-inactivated newborn calf serum and 0.2% so...
Table 1. Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>FITC Ab</th>
<th>PE Ab</th>
<th>CyC Ab</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1</td>
<td>Ms IgIGT</td>
<td>Ms IgGT</td>
<td>Ms IgGT</td>
<td>MS IgIGT</td>
</tr>
<tr>
<td>2  Kappa/AM</td>
<td>CD5/BD</td>
<td>CD5/BD</td>
<td>CD19/PH</td>
<td>MS IgIGT</td>
</tr>
<tr>
<td>3  Lambda/AM</td>
<td>CD5/BD</td>
<td>CD5/BD</td>
<td>CD19/PH</td>
<td>MS IgIGT</td>
</tr>
<tr>
<td>4  CD38/BD</td>
<td>CD22/CT</td>
<td>CD5/BD</td>
<td>CD19/PH</td>
<td>MS IgIGT</td>
</tr>
<tr>
<td>5  CD34/BD</td>
<td>CD11c/BD</td>
<td>CD5/BD</td>
<td>CD19/PH</td>
<td>MS IgIGT</td>
</tr>
<tr>
<td>6  CD10/GT</td>
<td>CD22/BD</td>
<td>CD5/BD</td>
<td>CD19/PH</td>
<td>MS IgIGT</td>
</tr>
<tr>
<td>7  CD10/GT</td>
<td>CD22/BD</td>
<td>CD5/BD</td>
<td>CD19/PH</td>
<td>MS IgIGT</td>
</tr>
<tr>
<td>8  HLA-DR/BD</td>
<td>CD56/BD</td>
<td>CD5/BD</td>
<td>CD19/PH</td>
<td>MS IgIGT</td>
</tr>
</tbody>
</table>

Abbreviations: GT, Gentrak (Plymouth Meeting, PA); PH, Pharmingen (San Diego, CA); AM, Amac (Westbrook, MA); BD, Becton Dickinson; CT, CalTag (South San Francisco, CA).

dium azide (PBS-NCS) and finally resuspended in 0.5 mL PBS-NCS for flow-cytometric analysis.

DNA content analysis. Pooled cells from triplicate wells were resuspended in 0.5 mL PBS containing 20 μg/mL propidium iodide (Sigma) and analyzed by flow cytometry.

Flow cytometry. Analysis was performed on a FACSScan (Becton Dickinson, Mountain View, CA). For immunophenotyping, light-scatter gating was used to eliminate dead cells and clumps. For each antibody tested, 30,000-count histograms of fluorescence intensity versus cell number were generated for the light-scatter-gated populations. For DNA content analysis, 10,000-count histograms were generated after doublet discrimination. Off-line data analysis of immunophenotyping and DNA content was performed using WinList and ModFit (Verity Software House, Topsham, MA), respectively.

RESULTS

PBMC from patients with either of two B-cell chronic leukemias (13 patients with CLL, 6 patients with HCL) were cultured with incremental concentrations of loxoribine in an attempt to induce cell-cycle entry. An unexpected selectivity of the substituted nucleoside for B-CLL cells was observed, with striking increases in DNA synthesis occurring in about three fourths (10 of 13) of B-CLL patients tested (Fig 1A). Leukemic B cells from patients with HCL, in contrast, failed to generate meaningful proliferative responses to loxoribine (Fig 1B). The clinical characteristics of the CLL patients studied are shown in Table 2. Flow cytometry showed that ≥94% of cells from all patients were CD5+, except for patient number 4 (85%) and patient number 9 (92%). Lack of correlation was found between stimulation index and absolute lymphocyte count (r = .174), Rai stage (r = .448), or time from diagnosis (r = .253). PB lymphocytes from normal donors have been reported to lack proliferative responsiveness to loxoribine.14 Uptake of [3H]TdR was assessed after 72-hour incubation. Kinetic studies showed this to be the time of peak stimulation (Fig 2). These latter studies also showed that the cells did not become irreversibly transformed, undergoing cycle upon cycle of mitosis; rather, [3H]TdR uptake subsided to baseline after a single peak of stimulation, despite the continued presence of the nucleoside in culture.

Morphologic confirmation of leukemic cell activation and cell-cycle entry was obtained by examination of cytocentrifuge preparations of cultured cells. The morphologic appearance of unstimulated, cultured B-CLL cells is shown in Fig 3A. Upon Wright-Giemsa staining, these cells manifest cytologic features typical of B-CLL, including small size, scant amounts of amphophilic cytoplasm, and relatively uniform

Fig 1. Differential induction of proliferative activity by loxoribine in chronic B cell leukemias. PBMC (10^6/mL) from patients with either CLL (A) or HCL (B) were cultured with incremental concentrations of loxoribine in a volume of 0.2 mL 10% heat-inactivated autologous plasma-containing medium for 72 hours. All cultures were pulsed with 0.6 μCi [3H]TdR for the final 24 hours of culture. Results for 13 CLL and 6 HCL patients are each presented as the arithmetic mean of [3H]TdR uptake per culture for five replicate cultures per point.
Table 2. Clinical Characteristics of CLL Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>ALC</th>
<th>Previous Treatment</th>
<th>Rai Stage at Time of Testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72/F</td>
<td>216,400</td>
<td>Chlor/Pred, 2CdA × 1</td>
<td>IV</td>
</tr>
<tr>
<td>2</td>
<td>69/F</td>
<td>141,670</td>
<td>CNOP; Chlor; CTX; VCR/Pred, 2CdA × 1</td>
<td>IV</td>
</tr>
<tr>
<td>3</td>
<td>78/M</td>
<td>32,280</td>
<td>None</td>
<td>IV</td>
</tr>
<tr>
<td>4</td>
<td>69/M</td>
<td>6,420</td>
<td>Chlor/Pred 86-89, CVP × 12 cycles</td>
<td>IV</td>
</tr>
<tr>
<td>5</td>
<td>52/M</td>
<td>9,830</td>
<td>Chlor 87-91, CVP × 3 cycles</td>
<td>IV</td>
</tr>
<tr>
<td>6</td>
<td>53/M</td>
<td>18,700</td>
<td>Multiple cycles of 2CdA</td>
<td>II</td>
</tr>
<tr>
<td>7</td>
<td>68/M</td>
<td>75,430</td>
<td>None</td>
<td>I</td>
</tr>
<tr>
<td>8</td>
<td>57/M</td>
<td>104,900</td>
<td>None</td>
<td>II</td>
</tr>
<tr>
<td>9</td>
<td>56/M</td>
<td>90,000</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>85/M</td>
<td>89,950</td>
<td>None</td>
<td>IV</td>
</tr>
<tr>
<td>11</td>
<td>44/M</td>
<td>157,250</td>
<td>VCR/Pred; 2CdA; Anti-CD19–ricin</td>
<td>IV</td>
</tr>
<tr>
<td>12</td>
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<td>Chlor/Pred, 2CdA × 4 cycles</td>
<td>II</td>
</tr>
<tr>
<td>13</td>
<td>64/F</td>
<td>229,000</td>
<td>Chlor/Pred; CVP; decacron; Ara-C/Carbo/Dexa; fludarabine</td>
<td>IV</td>
</tr>
</tbody>
</table>

Abbreviations: Chlor, chlorambucil; Pred, prednisone; CNOP, cytoxan, novantrone, oncovin, prednisone; CVP, cytoxan, vincristine, prednisone; VCR, vincristine; Ara-C/Carbo/Dexa, cytosine arabinoside/carboplatin/dexamethasone.

round nuclei containing coarse, clumpy chromatin and indistinct nucleoli. In contrast, loxoribine-activated B-CLL cells undergo dramatic cytologic changes. The activated cells show marked cellular enlargement and abundant basophilic cytoplasm with large nuclei containing evenly dispersed, fine, open chromatin and single, prominent nucleoli (Fig 3B). Occasional mitotic figures are also seen (Fig 4). The activated lymphocytes morphologically resemble prolymphocytes in many respects.

Parallel cultures were stained for tartrate-resistant acid phosphatase activity (TRAP), in consideration of the partial HCL-like morphologic and histochemical conversion that B-CLL cells are described to undergo upon exposure to phorbol esters.9 No induction of TRAP activity (red punctate staining) and no morphologic changes suggestive of HCL are noted when comparing unstimulated (Fig 3C) with activated (Fig 3D) cells. Positive controls were included with all TRAP stains. Nucleolar size changes are particularly striking with this stain.

Loxoribine and its analogues are well-characterized inducers of both proliferation and functional differentiation to Ig secretion in murine B cells in vitro.15-19 Immunohistochemical stains for cytoplasmic Ig were performed, to determine if loxoribine also induced Ig production in B-CLL cells. Comparison of anti-κ staining of loxoribine-activated B-CLL cells (Fig 3F) with identically-stained unstimulated control cells (Fig 3E) clearly indicates that the loxoribine-treated cells exhibit increased cytoplasmic staining for κ-light chain.

The effects of loxoribine-dependent B-CLL cell activation on cell surface antigen expression were explored. B-CLL cells were cultured with incremental concentrations of loxoribine for 72 hours, reacted with monoclonal antibodies specific for an array of surface determinants, and analyzed by flow cytometry. The data indicate that dramatic upregulation of CD22, CD23, CD25, CD38, and CD54 expression occurred, although with differing dose optima (Fig 5, upper panels). Interestingly, a very high proportion of cells expressed these antigens at low levels without stimulation, except for CD38, whose intermediate level expression increased to involve the great majority of cells upon activation. Marginally increased expression of CD1ic and surface λ-light chain was noted, with no significant change in CD5, HLA-DR, or CD20 staining intensity (Fig 5, lower panels). A modest increment in the proportion of cells expressing CD20 (increasing from 5% to 35%) was observed. These findings were representative of all four patients for whom cell surface phenotypes were studied. Flow-cytometric analysis of cells from the patient of Fig 5 stained with propidium iodide indicated that 2% of control cells
Fig 3. Morphology of loxoribine-activated B-CLL cells. Cells were cultured in the presence or absence of optimal concentrations of loxoribine for 3 days and were then transferred to microscope slides with a cytocentrifuge. Cells were cultured with medium alone (A, C, and E), or with loxoribine (B, D, and F). (A) and (B) are stained with Wright-Giemsa stain; (C) and (D) are stained with the tartrate-resistant acid phosphatase procedure; and (E) and (F) are stained with alkaline-phosphatase-conjugated anti-κ antibodies.

and 11% of loxoribine-stimulated cells were in S + G2/M.

The success of a therapeutic regimen that first induces entry of malignant cells into the cell cycle and subsequently enlists cycle-active cytotoxic drugs to kill them, will depend upon the ability of cells from such patients to respond to stimulation after repeated rounds of cytotoxic therapy. Therefore, cells from a patient undergoing treatment for CLL with 2-chloro-2'-deoxyadenosine (2CdA) were obtained before initiation of treatment, and after each of his cycles of 2CdA. After one and two cycles of 2CdA (sufficient to lower the patient’s peripheral white blood cell [WBC] count from 94 × 10⁹/L to 10 × 10⁹/L) the patient’s cells retained excellent stimulability by loxoribine in vitro relative to his initial pattern of responsiveness (Fig 6).

In other studies, B-CLL cells were cultured with loxoribine for two days to allow cells to achieve optimal degrees of sensitization before addition of various cytotoxic drugs. Three weeks thereafter, the number of viable cells per milliliter was determined. The cell count remained significantly elevated in loxoribine-activated cultures, as compared with control cultures. Dexamethasone, a noncycle active agent, failed to decrease the number of viable cells relative to control, although it did counter loxoribine-dependent proliferation (Fig 7A). In contrast, doxorubicin and VP-16, both cycle-active cytotoxic agents, exhibited synergistic killing of CLL cells, despite the fact that neither one alone induced significant cytotoxicity in the absence of loxoribine. The ability of loxoribine to sensitize CLL cells from six other patients to a variety of antileukemia drugs is shown in Fig 7B.

DISCUSSION

In B-CLL, the malignant B cell clone has been thought to be developmentally arrested at a stage of differentiation between pre-B-cell and mature-B-cell stages. The relative chemosensitivity and incurability of this disease is attributable largely to the fact that these cells are primarily small, resting Go- or G0/G1-phase cells that rarely divide and only gradually accumulate. Clinically, this is reflected in the indolent nature of a disease characterized by a long latent period. Stimulation of B-CLL cells in vivo to a state of active proliferation by a pharmacologic
ACTIVATION OF B-CLL CELLS BY LOXORIBINE

Modulation of surface antigens on CLL cells by Loxoribine

Fig 5. Modulation of surface antigens on B-CLL cells by loxoribine. PBMC (10⁶/mL) from B-CLL patients were cultured in the presence of incremented concentrations of loxoribine for 3 days. At that time, all cultures were processed and stained for cell surface antigens as described in Materials and Methods and analyzed by flow cytometry. Results are presented as mean fluorescence intensity (---) and percent positive cells (—).
DNA synthesis and differentiation in B-CLL cells from a patient with an IgM paraprotein, using pokeweed mitogen (PWM) with allogeneic T cells. Low-level responses were induced by other investigators using anti-\( \mu \) antibodies, SAC, calcium ionophore, PHA, purified protein derivative of tuberculin (PPD), PWM, Epstein-Barr virus, lipopolysaccharide, and dextran sulfate. In 1980, Totterman et al.

Evidence to date suggests that activation of B-CLL cells by loxoribine uses a pathway distinct from that used by phorbol esters. Thus, phorbol esters induce increased cell surface expression of CD5 and class II MHC antigens; loxoribine has no effect on the former and only a sporadic effect on the latter. Phorbols down-regulate CD20, CD21, sIgM, and sIgD; loxoribine induces none of these effects. Whereas TPA has been found to induce TRAP positivity in B-CLL cells, loxoribine fails to do so. Phorbols initiate translocation and activation of cytosolic protein

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**Fig 6.** Proliferation of B-CLL cells in response to loxoribine before and after in vivo chemotherapy. PBMC (10^6/mL) from a patient with B-CLL were cultured as in Fig 1, with incremental concentrations of loxoribine. The identical experiment was repeated (with fresh plasma for medium each time) before the second and third cycles of chemotherapy with infusional 2CdA. Results are presented as in Fig 1. (□) Before first cycle of 2-CdA; (■) before second cycle of 2-CdA; (△) before third cycle of 2-CdA.

Loxoribine concentration [\( \mu M \)]
kinase C; in normal murine B cells, loxoribine bypasses protein kinase C. Finally, loxoribine alone induces S phase entry, transformation, and mitosis in a discrete proportion of B-CLL cells; phorbol esters do not consistently do so.

The implications of these observations in the therapeutic armamentarium of the chronic low-grade lymphoid neoplasms are clear—the potential for inducing malignant lymphocytes into sensitive phases of the cell cycle overcomes a form of resistance to therapy, may reversibly transform a low-grade lymphocytic malignancy to a higher grade, and may thereby add significantly to the development of curative strategies for these incurable chronic neoplastic conditions.

REFERENCES


Fig 7. Sensitization by loxoribine of B-CLL cells to killing by cytotoxic drugs. (A) PBMC (10^6/mL) from the patient of Fig 6 were cultured for 48 hours with or without optimal concentrations of loxoribine before addition of cytotoxic drugs: 1 μmol/L dexamethasone, 1μmol/L doxorubicin, 10 μg/mL VP-16, or a combination of all three. Viabilities were evaluated after 21 days in culture. (B) Cells from six other patients were cultured for 48 hours with or without optimal concentrations of loxoribine before addition of cytotoxic drugs: patient 1, doxorubicin (assayed on day 6); patient 2, vincristine (day 6); patient 3a, doxorubicin (day 6); patient 3b, Ara-C (day 9); patient 4, VP-16 (day 14); patient 5, VP-16 (day 6); patient 6, doxorubicin (day 3).
PHOTOCYTOTES. Science 234:697, 1986


Loxoribine induces chronic lymphocytic leukemia B cells to traverse the cell cycle

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