Sequential Treatment of a Resistant Chronic Lymphocytic Leukemia Patient with Bryostatin 1 Followed by 2-Chlorodeoxyadenosine: Case Report

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ABSTRACT

Bryostatin 1 (Bryo-1) has been shown to differentiate chronic lymphocytic leukemia (CLL) cells to the hairy cell leukemia phenotype. The purine analogue 2-chlorodeoxyadenosine (2-CdA) exhibits enhanced activity in patients with hairy cell leukemia compared to those with CLL. Here we present a case report of a patient diagnosed with resistant CLL and treated sequentially with Bryo-1 followed by 2-CdA for three cycles. Molecular and biochemical parameters relative to the sequential treatment with these agents in vivo were comparable to those found in the WSU-CLL cell line in vitro (R. M. Mohammad et al., Clin. Cancer Res., 4: 445–453, 1998; R. M. Mohammad et al., Biol. Chem., 379: 1253–1261, 1998). There was a significant reduction of lymphocyte count from 37.1 × 10^9/µl before the treatment to 3.4 × 10^9/µl after treatment, and partial remission was achieved 2 months after the treatment. The percentage of morphologically differentiated lymphocytes was increased from 3% before treatment to 92% with the first cycle of Bryo-1. Similarly, expression of CD22, a marker of differentiation, increased from 38% to 97% and was maintained at a high level for the duration of the treatment. Analysis of the molecular markers of apoptosis in isolated peripheral blood lymphocytes revealed an increase in the Bax:Bcl-2 ratio after treatment with Bryo-1 in cycles 2 and 3, with associated poly(ADP-ribose) polymerase cleavage after Bryo-1 and 2-CdA treatment. The deoxycytidine kinase: cytosolic 5′-nucleotidase activity ratio increased modestly after Bryo-1 treatment, indicating increased sensitivity of the peripheral blood lymphocytes to 2-CdA. In summary, we found that sequential treatment with Bryo-1 and 2-CdA caused a significant reduction in peripheral blood lymphocytes (CLL cells) with simultaneous induction of differentiation and the initiation of the Bax:Bcl-2 apoptotic pathway.

INTRODUCTION

CLL is the most common adult leukemia in Western countries. Alkylating agents, corticosteroids, and the new purine analogues are the standard treatment for CLL. Over time, patients become resistant to these therapeutic agents, and further treatment options are limited. Because there is no curative therapy for CLL, the discovery of antileukemic agents with novel mechanisms of action and their subsequent incorporation into combination regimens may lead to improved outcome of this disease. Bryo-1, a macrocyclic lactone, is a partial PKC activator that has differentiating activity in CLL of B-cell origin (1, 2). It is extracted and purified from the marine animal Bugula neritina (3). The differentiation of CLL to HC is based on the induction of HC-associated markers such as CD11c, CD22, and tartrate-resistant acid phosphatase. 2-CdA is a purine analogue that is very active in HCL but is inactive in patients with resistant CLL (4, 5). In the WSU-CLL cell line, using different combinations of exposure to Bryo-1 and 2-CdA, only sequential exposure to Bryo-1 for 72 h followed by 2-CdA for another 72 h resulted in significant growth inhibition and apoptosis (6). In vitro schedule and resulting effects can be reproduced using a xenograft model of WSU-CLL in severe combined immunodeficient (SCID) mice. The data on survival in days, tumor growth inhibition, tumor growth delay, and the log_{10} kill in mice treated with Bryo-1 followed by 2-CdA were all significantly better than those found in the untreated control groups or in the group of animals treated with Bryo-1 alone or 2-CdA alone (6). The increased ratio of dCK:5′-NT activity and the increased Bax:Bcl-2 ratio are at least two mechanisms through which this natural product is able to potentiate the antitumor activity in previously resistant WSU-CLL cells. Hence, our previous preclinical studies using the WSU-CLL cell line in culture and a xenograft model of CLL in SCID mice provided the rationale for using sequential treatment of Bryo-1 followed by 2-CdA in patients with CLL.

Here we report the laboratory findings in a single patient with CLL in a Phase I clinical trial. This trial was designed to treat patients with Bryo-1, followed by 2-CdA. We examined the change in cell differentiation and the molecular pathways of

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The abbreviations used are: CLL, chronic lymphocytic leukemia; Bryo-1, bryostatin 1; HC, hairy cell; HCL, HC leukemia; 2-CdA, 2-chlorodeoxyadenosine; dCK, deoxycytidine kinase; 5′-NT, 5′-nucleotidase; PKC, protein kinase C; PARP, poly(ADP)ribose polymerase.
cell death by apoptosis associated with sequential treatment of Bryo-1 and 2-CdA in a patient.

MATERIALS AND METHODS

Case Report. A 69-year-old man diagnosed with CLL in 1991 was referred to our clinic in November 1998 because of an increase in WBC count. He received different chemotherapeutic regimens including chlorambucil, prednisone, and fludarabine. He denied any symptoms of fever, night sweat, or weight loss. He had a history of basal cell carcinoma, which had been removed from his back. He smoked a pipe for many years and occasionally smoked cigars and was a frequent user of alcohol. He was employed as a toolmaker and had been exposed to asbestos. On physical examination, he had palpable bilateral small inguinal nodes and no palpable supraclavicular or axillary nodes. His liver or spleen was not palpable. Patient’s complete blood count was as follows: WBC, 52 × 10^3/μl; granulocytes, 3.6 × 10^3/μl; lymphocytes, 47.6 × 10^3/μl; hemoglobin, 12.3 g/dl; hematocrit, 36.2%; and platelets, 98 × 10^3/μl. The patient was reviewed in the Multi-Disciplinary Lymphoma/Leukemia Clinic, and his blood smear slides were examined to reconfirm the diagnosis of CLL. Because there was a rapid increase in WBC despite the maintenance of previous chemotherapy regimens (doubling of lymphocytes within 3 months), the patient was enrolled in the Bryo-1/2-CdA study after signing the informed consent form. The patient was treated with three cycles of Bryo-1 and 2-CdA from November 1998 to January 1999.

Chemotherapy Protocol. Bryo-1 (252 μg; 120 μg/m²) in 0.9% benzo-therapy-preserved saline was infused through infusion-ports for 72 h followed by 2-CdA (26.4 mg; 0.06 mg/kg) by continuous infusion over 5 days. The patient underwent three cycles of chemotherapy during November 17–25, 1998; December 15–23, 1998; and January 19–27, 1999. During each cycle, blood samples were collected for the isolation of lymphocytes at the following time points: before Bryo-1 administration (pre-Bryo); at the completion of Bryo-1 administration before 2-CdA administration (post-Bryo); and at the completion of 2-CdA administration (post-2CdA). Bryo-1 (NSC 339555) was supplied by the National Cancer Institute Division of Cancer Treatment and Diagnosis.

Peripheral Blood Mononuclear Cells. Blood samples were collected at the time points indicated above via venipuncture in heparinized tubes. Lymphocytes were isolated by a density gradient using Histopaque (Sigma Diagnostics Inc., St. Louis, MO). The lymphocyte layer was recovered and washed density gradient using Histopaque (Sigma Diagnostics Inc., St. Louis, MO). The lymphocyte layer was recovered and washed density gradient using Histopaque (Sigma Diagnostics Inc., St. Louis, MO). The lymphocyte layer was recovered and washed with PBS, resuspended in Triton X-100 lysis buffer, and handled as described previously (7). For Western analysis, 20 μg from each sample were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked and then incubated in 1:1000 concentration of Bax, Bcl-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and PARP (Travigen, Gaithersburg, MD) antibodies. The membranes were exposed to horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.). The proteins were visualized using the enhanced chemiluminescence reagents (Amersham International Ltd., Buckinghamshire, United Kingdom).

dCK. Patient lymphocytes were analyzed at the pre-Bryo and post-Bryo time points for dCK activity using 2-CdA as a substrate, as described previously (7). Cells were harvested, washed once with PBS, and resuspended at a concentration of 10 × 10^6 cells/ml in dCK sonication buffer consisting of 10 mM Tris·HCl (pH 7.6), 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Samples were stored at −70°C until assayed.

Cytosolic 5’-NT Activity. Isolated patient lymphocytes were washed once with PBS and resuspended at a concentration of 10 × 10^6 cells/ml in 5’-NT sonication buffer consisting of 20 mM imidazole-HCl (pH 7.0), 20 mM MgCl₂, 0.1 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride. Samples were stored at −70°C until assayed as described previously (7). Results are presented as a ratio of the dCK activity (expressed as pmol/mg protein/min): 5’-NT activity (expressed as pmol inosine monophosphate diphosphorylated/mg protein/min).

RESULTS

Lymphocyte Morphology. The percentage of differentiated lymphocytes in isolated peripheral blood lymphocytes by light microscopy was determined as described previously (2). We examined the proportion of differentiated and nondifferentiated cells among these viable cells. Nondifferentiated cells are smaller in size and have a low nuclear:cytoplasmic ratio, whereas the differentiated cells are relatively larger in size with cytoplasmic processes and a high nuclear:cytoplasmic ratio.

Expression of Differentiation Cell Markers. Peripheral blood mononuclear cells from the patient were obtained by ficoll-hypaque separation at pre-Bryo, post-Bryo, and post-2CdA time points of each chemotherapy cycle. Cells were stained simultaneously with three antibodies directly conjugated to peridinin chlorophyll protein (CD20), phycocerythrin, and FITC. CD20+ cells were gated as total B cells of interest, within which the proportion of cells that expressed CD5 alone (CD20+, CD5+) or with CD11c (CD20+, CD5+, CD11c+) or CD22 (CD20+, CD5+, CD22+) were evaluated.

Western Blot Analysis. The patient’s isolated lymphocytes were washed with PBS, resuspended in Triton X-100 lysis buffer, and handled as described previously (7). For Western analysis, 20 μg from each sample were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked and then incubated in 1:1000 concentration of Bax, Bcl-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and PARP (Travigen, Gaithersburg, MD) antibodies. The membranes were exposed to horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.). The proteins were visualized using the enhanced chemiluminescence reagents (Amersham International Ltd., Buckinghamshire, United Kingdom).
The peripheral blood, bone marrow examination showed more lymphadenopathy and hepatosplenomegaly. Unfortunately, all, respectively. After the first cycles of Bryo-1 and 2-CdA administration (Table 1). Before and the lymphocyte count were monitored during the three cycles.

During the second and third cycle, CD22-positive cells were 92%, and double-positive cells for CD11c and CD22 remained the same. At the post-2CdA time point, CD22-positive cells were increased to 93%, 4% of cells were double positive for CD11c and CD22, respectively. In the first cycle of treatment, at the pre-Bryo time point of cells among the viable cells on the specified time point.

**Expression of Differentiated Cell Markers.** The expression of cell surface markers (CD11c+ /CD22+, CD11c+ /CD22+, and CD22+ ) in lymphocytes of this patient was evaluated by two-color flow cytometry after the first, second, and third cycle. In the first cycle of treatment, at the pre-Bryo time point, 4% of cells were double positive for CD11c and CD22, and 34% of cells were CD22 positive (Table 1). At the post-Bryo time point, CD22-positive cells were decreased to 93%, whereas the percentage of double-positive cells for CD11c and CD22 remained the same. At the post-2CdA time point, CD22-positive cells were 92%, and double-positive cells for CD11c and CD22 were 5%. During the second and second cycle, CD22-positive cells remained elevated from 76% to 90%, and the double-positive cells for CD11c and CD22 were elevated from 6% to 8%.

**Clinical Response.** Peripheral blood total WBC count and the lymphocyte count were monitored during the three cycles of Bryo-1 and 2-CdA administration (Table 1). Before the onset of treatment, the total WBC count and lymphocyte count were 60,500 and 37,100/μl, respectively. After the first cycle, the total WBC count was reduced to 26,000/μl and remained decreased for up to 2 months after the third cycle. Lymphocyte count was also reduced to 5,940/μl after first cycle and gradually declined thereafter. According to the National Cancer Institute Working Group criteria (8), the patient achieved partial remission. The hemoglobin, neutrophil, and platelet counts were maintained above 11 gm/dl, 1.5 × 10^9/liter, and 100 × 10^9/liter, respectively, and the patient was free of lymphadenopathy and hepatosplenomegaly. Unfortunately, although there was a significant reduction in lymphocyte count in the peripheral blood, bone marrow examination showed more than 30% lymphocytes, which precluded a designation of complete remission. The patient’s absolute lymphocyte count increased to >10 × 10^9/liter 2 months after the discontinuation of therapy.

**Western Blot Analysis.** The gradual increase in the Bax: Bcl-2 ratio from 0.90 in first cycle to 7.5 in second cycle and 14.3 in the third cycle was primarily due to the reduction of Bcl-2 expression and an increase in Bax expression (Fig. 2). In all three cycles, there was a relative reduction of Bcl-2 expression compared to the internal control glyceraldehyde-3-phosphate dehydrogenase as determined by densitometric analysis of the Western blots. At the post-Bryo time point of three cycles, the relative expression of Bcl-2 was 1.94, 0.18, and 0.09, respectively. In the first cycle, PARP (116 kDa) was significantly reduced post-Bryo when compared to that recorded in the pre-Bryo. The profile of PARP in the second cycle did not appear to change except for further change of the 116-kDa band to 85-kDa band by post-2CdA. In the third cycle, post-Bryo, PARP cleavage was complete, with no detectable 116- or 85-kDa bands. Post-2CdA showed significant cleavage of the PARP 116-kDa to 85-kDa bands.

**Ratio of dCK: 5′-NT activity.** The ratio of dCK: 5′-NT activity in peripheral blood lymphocytes, for all three cycles, at pre-Bryo and post-Bryo time points, is shown in Fig. 3. Post-2CdA was excluded because dCK uses 2-CdA as its substrate. In the first, second, and third cycle, at post-Bryo, the dCK: 5′-NT ratio was higher than the pre-Bryo ratio, but there were no statistical differences between the means.

**DISCUSSION**

We have been able to demonstrate in this patient that infusion of Bryo-1 for 72 h induces peripheral blood lymphocyte HC morphology and increases the sensitivity of cells to 2-CdA. Moreover, there was a significant reduction of lymphocyte count from 37.1 × 10^3/μl before the treatment to 3.4 × 10^3/μl, and partial remission was achieved 2 months after the treatment. Sequential treatment with Bryo-1 followed by 2-CdA induced the initiation of apoptosis.

Bryo-1 has been reported to induce differentiation of CLL cells of B-cell origin to a HC stage in vitro (2). We have previously documented that Bryo-1-treated CLL cells exhibit increased sensitivity to 2-CdA, a drug active in treating de novo HCL but not active in fludarabine-resistant CLL. In WSU-CLL cells in vitro as well as the xenograft model in SCID mice, the efficacy of 2-CdA was enhanced when the cells were first exposed to Bryo-1 (6, 7). Administration of Bryo-1 followed by 2-CdA appeared to be sequence-dependent because neither concurrent nor reverse sequential use of the two agents was more effective than either agent alone (6).

Bryo-1 is a potent modulator of PKC, which elicits a wide range of biological effects including in vitro and in vivo antineoplastic activity, induction of differentiation, pleotropic immuno-enhancing effects on both T and B lymphocytes, activation of polymorphonuclear leukocytes, and enhancement of the production and function of hematopoietic growth factors (9). Bryo-1-induced translocation of PKC to the plasma membrane leads to the phosphorylation of specific protein substrates and eventually induces down-regulation of PKC (10). In patients, activation of...
PKC activity is increased within first couple of hours after treatment with Bryo-1 and is then subsequently down-regulated by 24 h on (11).

We have demonstrated here that Bryo-1 infusion for 72 h induced differentiation of the CLL cells (Fig. 1), as evidenced by the presence of cell membrane processes and increased nuclear/cytoplasmic ratio. By using flow cytometry, we further showed that differentiated cells bear HC-associated surface markers CD22 and CD11c (Table 1). Differentiation was most evident after the first cycle of Bryo-1 treatment. Despite subsequent administration of Bryo-1, we did not observe a marked change in cell differentiation in the second and third cycle compared to that found in the first cycle, possibly because of an accumulation of Bryo-1-resistant CLL cells over time. These observations confirm that Bryo-1 induces differentiation in peripheral blood lymphocytes in vitro similar to that demonstrated using the WSU-CLL cell line in vitro and in vivo using a SCID mouse xenograft model.

Among the purine analogues, 2-CdA has the ability to induce apoptosis in nondividing lymphocytes at concentrations that spare other cell types (12). For this reason, 2-CdA is used in the treatment of indolent lymphoid malignancies, including HCL and low-grade lymphoma. The cytotoxicity of 2-CdA depends mainly on its selective and progressive phosphorylation by dCK and accumulation of its 5'-triphosphate metabolite (2-CdATP) in lymphocytes. 2-CdAMP can be dephosphorylated back to the nucleoside by a cytosolic 5'-NT. Hence, both the in vitro sensitivity of cultured CLL cells and the in vivo response of patients with CLL to 2-CdA correlate with the high ratio of dCK; 5'-NT (12–14). Bryo-1 increases the sensitivity to 2-CdA by increasing the dCK; 5'-NT ratio (7) and by increasing intracellular incorporation of 2-CdA (13). The phosphorylated metabolite of 2-CdA, 2-CdATP, is a potent inhibitor of ribonucleotide reductase and DNA polymerase α, and these two enzymes are responsible for DNA synthesis and repair in actively dividing cells (15, 16). Inhibition of the repair mechanism can lead to DNA fragmentation, which is characteristic of apoptosis in dividing cells.

PARP cleavage is an accepted indicator for the initiation of apoptosis. PARP is a 116-kDa nuclear protein that maintains DNA repair pathways. Once it is cleaved into 85- and 22-kDa fragments, the DNA repair mechanism is impaired, which ultimately leads the cells to undergo apoptosis. Hence, the presence of the 85-kDa band is an indicator for PARP cleavage. In peripheral mononuclear cells, the sequential treatment with Bryo-1 and 2-CdA led to an increase in the Bax:Bcl-2 ratio as well as evidence of PARP cleavage (Fig. 2). This indicates that the signal transduction pathways leading to apoptosis have been

### Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>First cycle</th>
<th>Second cycle</th>
<th>Third cycle</th>
</tr>
</thead>
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<tr>
<td></td>
<td>pre-Bryo</td>
<td>post-Bryo</td>
<td>post-2CdA</td>
</tr>
<tr>
<td>CD11c+/CD22+</td>
<td>1500 (4%)</td>
<td>240 (4%)</td>
<td>300 (5%)</td>
</tr>
<tr>
<td>CD11c-CD22+</td>
<td>12500 (34%)</td>
<td>5520 (93%)</td>
<td>5500 (92%)</td>
</tr>
<tr>
<td>CD22</td>
<td>23100 (62%)</td>
<td>200 (3%)</td>
<td>180 (3%)</td>
</tr>
<tr>
<td>Total lymphocytes</td>
<td>37100</td>
<td>5940</td>
<td>5980</td>
</tr>
<tr>
<td>WBC</td>
<td>60500</td>
<td>26000</td>
<td>24030</td>
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Fig. 2 Analysis by Western blot indicated a gradual increase in the Bax:Bcl-2 ratio after Bryo-1 treatment: Bax and Bcl-2 expression was calculated as the Bax:Bcl-2 ratio to the loading control (G3PDH) expression. Evidence of cleavage of PARP could be seen after sequential treatment with Bryo-1 and 2-CdA.

Fig. 3 There was an increase in the ratio of dCK; 5'-NT activity after Bryo-1 infusion. dCK and 5'-NT values are expressed as pmol/mg protein/min. However, this increase was not statistically significant. Post-2CdA was excluded because dCK uses 2-CdA as its substrate.
initiated in these peripheral blood lymphocytes. In vitro, studies have shown that the activation of caspase 3 precedes PARP cleavage and is easily detected by Western blot analysis (17). We did not observe caspase 3 or cleavage in the peripheral blood lymphocytes isolated from this patient (data not shown). The significant reduction of total WBC count and lymphocyte count during the treatment and 2 months after the termination of treatment provides evidence for the effectiveness of sequential treatment of Bryo-1 and 2-CdA in eliminating malignant CLL cells by differentiating the cells to HC and inducing apoptosis.

In this patient, the dCK:5′-NT ratio was elevated after 3 days of infusion of Bryo-1. This suggests that the Bryo 1-induced elevation of the dCK:5′-NT ratio may lead to increased sensitivity of peripheral blood lymphocytes of this patient to 2-CdA. The increased sensitivity to 2-CdA of the peripheral blood lymphocytes induced by Bryo-1 is reflected by the elevated dCK:5′-NT ratio at the post-Bryo time point. Hence, to further understand the efficacy of 2-CdA, peripheral blood lymphocytes of the patient should be analyzed for dCK and 5′-NT activity 1 day or 2 days after the initiation of the 5-day infusion of 2-CdA.

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