Sequential Treatment of Human Chronic Lymphocytic Leukemia with Bryostatin 1 followed by 2-Chlorodeoxyadenosine: Preclinical Studies

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ABSTRACT

We have previously reported that bryostatin 1 (Bryo 1) induces differentiation of chronic lymphocytic leukemia (CLL) in vitro to a hairy cell (HC) stage. This study tests the hypothesis that Bryo 1-differentiated CLL cells are more susceptible to 2-chlorodeoxyadenosine (2-CdA) than parent CLL cells. A recently established EBV-negative CLL line (WSU-CLL) from a patient resistant to chemotherapy including fludarabine was used to test this hypothesis. Both Bryo 1 (10–1000 nM) and 2-CdA (5.6–22.4 μM) exhibited a dose-dependent growth inhibitory effect on the WSU-CLL cell line. In vitro, the sequential exposure to Bryo 1 (100 nM for 72 h) followed by 2-CdA (11.2 μM) resulted in significantly higher rates of growth inhibition than either agent alone. Changes in immunophenotype, enzymes, lipids, proteins, and the DNA of WSU-CLL cells were studied before and after Bryo 1 treatment. Bryo 1 induced a positive tartrate-resistant acid phosphatase reaction and two important markers, CD11c and CD25, after 72 h of culture, confirming the differentiation of CLL to HC. The Fourier transformation infrared spectroscopic analysis showed that the amount of membrane lipids significantly increased in Bryo 1-treated cells compared to controls after 24 h, whereas the protein content, as well as the DNA content, decreased. This finding supports the change of CLL to HC. To evaluate the in vivo efficacy of Bryo 1 and 2-CdA, we used a xenograft model of CLL in WSU-CLL-bearing mice with severe combined immune deficiency. s.c. tumors were developed by injection of 10⁶ WSU-CLL cells, and fragments were then transplanted into a new batch of severe combined immunodeficient mice. Bryo 1 and 2-CdA at the maximum tolerated doses (75 μg/kg i.p. and 30 mg/kg s.c., respectively) were administered to the mice at different combinations and schedules. The survival in days, the tumor growth inhibition ratio, the tumor growth delay, and the log₁₀ kill of the mice treated with Bryo 1 followed by 2-CdA were significantly better than the control and other groups. We conclude that the sequential treatment with Bryo 1 followed by 2-CdA resulted in higher antitumor activity and improved animal survival.

INTRODUCTION

B-cell CLL is the most common adult leukemia, accounting for approximately 30% of all cases in Western countries (1). When therapy is required, alkylating agents, steroids, and, more recently, the new purine analogue, fludarabine, are used. Over time, patients become resistant to these agents, and treatment options at this point are limited. 2-CdA is a purine analogue that is very active in patients with HC leukemia but inactive in patients with fludarabine-resistant CLL (2). There is no curative therapy for CLL, and there has been little success in extending the overall duration of survival. The discovery of antilymphoid agents with novel mechanisms of action and their subsequent incorporation into combination regimens should improve the outcome in this disease.

As part of the National Cancer Institute Natural Products Program, a number of novel agents with antilymphoid activity, derived from marine products, have been identified and are now entering clinical trials. Also, among new treatment modalities is the use of biological agents in combination with standard chemotherapeutic agents. Bryo 1, a macrolycyclic lactone and a protein kinase C activator that has a differentiating activity on CLL tumors (3), is extracted and purified from the marine animal Bugula neritina (4). It has the ability to differentiate CLL cells to a HC stage (5). This differentiation is based on morphological changes and on the induction of HC-associated markers like CD11c and TRAP. This study tested the hypothesis...

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1 The abbreviations used are: CLL, chronic lymphocytic leukemia; 2-CdA, 2-chlorodeoxyadenosine; Bryo, bryostatin; HC, hairy cell; TRAP, tartrate-resistant acid phosphatase; PT-IR, Fourier transformation infrared; SCID, severe combined immunodeficient; AcP, acid phosphatase; MTD, maximum tolerated dose; WSU, Wayne State University.
that Bryo 1-differentiated CLL cells are more susceptible to 2-CdA than parent CLL cells.

FT-IR spectroscopy has become a powerful tool for the analysis of cellular components and also for complex biological samples, such as body fluids, tissues, or cell cultures (6–8). For example, using FT-IR spectroscopy, purified B-CLL cells from patients can be distinguished from those of normal peripheral blood by making use of two IR marker bands, at 966 and 530 cm⁻¹, and/or the area ratio of two spectral regions, at 1080 and 1540 cm⁻¹. Recently, Schultz et al. (9) were able to demonstrate qualitative and quantitative differences in DNA, proteins, and lipids between CLL cells and normal lymphocytes. The same group also used the differences in the FT-IR spectra of CLL cells from patients to predict the ex vivo sensitivity/resistance to treatment with 2-CdA and chlorambucil (10) and concluded that 2-CdA-resistant CLL cells express FT-IR spectra differently from 2-CdA-sensitive CLL cells. In the present study, we examined the variation observed in the FT-IR spectra of WSU-CLL cells before and after treatment with Bryo 1.

We report here the preclinical activity of Bryo 1 followed by 2-CdA in B-CLL, using a SCID mouse xenograft model bearing a CLL line (WSU-CLL; Ref. 11). We also demonstrated, by using FT-IR spectroscopy, that the in vitro treatment of the WSU-CLL cells with Bryo 1 induces changes in lipids, proteins, and DNA consistent with cells sensitive to 2-CdA or to those having the features of HC leukemia.

MATERIALS AND METHODS

In Vitro Studies. The WSU-CLL cell line was established in our laboratory from a 66-year-old male with CLL refractory to cyclophosphamide, vincristine, and prednisone; fludarabine; and vincristine, Adriamycin, and dexamethasone. The cell line shows multiple chromosomal aberrations with 45, x, del (3)(p14;p24), t(4;12)(q31;q22;p13), t(5;12)(q31; p13), add(16)(q24)x2, t(18;21)(q12;p12). It grows in liquid culture and forms s.c. tumors in SCID mice. The WSU-CLL cell suspension was maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 1% l-glutamine, 100 unit/ml penicillin G, and 200 μg/ml streptomycin at a density of 2 × 10⁶ cells/ml. Cells were seeded at a concentration of 2 × 10⁵/ml in the culture medium and were treated concurrently or sequentially with Bryo 1 (generously provided by G. R. Pettit, Arizona State University) at concentrations of 100, 200, and 1000 nm or 2-CdA (OrthoBiotech, Inc., Raritan, NJ) at concentrations of 5.6, 11.2, 16.8, and 22.4 μM. To an equal number of wells, diluent (0.02% DMSO) was added to the medium and used as control. Cells were counted daily for 5 days using trypan blue dye and hemocytometer. The entire experiment was repeated three times.

Immunofluorescence Staining and Flow Cytometry. Cells to be stained were aliquoted into 75-mm tubes at a concentration of 5 × 10⁶ cells/tube and washed in 3 ml of PBS with 1% BSA as described previously (12). Monoclonal antibodies (mouse antihuman) to B cell differentiation antigens or to immunoglobulins were added at saturating concentrations, mixed, and incubated at 4°C for 35 min. Cells were washed again and incubated for 30 min at 4°C with a second antibody (goat antimouse) that was FITC-conjugated. Finally, cells were washed and resuspended in 0.5 ml of 1% BSA in PBS and analyzed on a FACScan flow cytometer (Beckon Dickinson). A minimum of 20,000 cells per test were analyzed. The results were given as a percentage of cells expressing immunofluorescence. For the dual color staining, cells were incubated with combinations of FITC- and phycoerythrin-conjugated monoclonal antibodies at 4°C for 30 min in the dark. Mouse immunoglobulin isotypes conjugated with FITC or phycoerythrin were used as a negative control. Results were expressed as a percentage of cells positive for single or double markers.

Enzymatic Studies. Cytocentrifuge smears were prepared from the control and Bryo 1-treated cultures using the Cytospin II centrifuge (Shandon, Southern Instruments, Inc., Sewickley, PA). The air-dried smears were fixed in 60% acetone containing 3% glutaraldehyde and then stained for AcP with and without tartrate, according to Hayhoe and Flems (13).

IR Spectroscopic Analysis of WSU-CLL Cells. WSU-CLL cells from control and Bryo 1-treated cultures after 24 h were washed and resuspended in 0.9% NaCl. Five μl of the suspensions (~3 × 10⁶ cells) were deposited on IR-transparent BaF₂-windows and dried down as thin circular disc films with a diameter of 2–3 mm. The films were kept in a desiccator under a mild vacuum for 3–5 min. The BaF₂-window was used to stabilize the humidity of the films. The quality of each preparation was checked by IR microscopy within the sampling area of 14 mm². This methodology produced good homogenous films, free of contaminating chemicals. For IR spectra and cluster analysis, the same method was used as described previously (9, 10). Briefly, for all samples, three spectra, each from a different film, were obtained against a blank background. The IR measurements were carried out on a FTS-60 FT-IR spectrometer (Bio-Rad, Cambridge, MA), equipped with a nitrogen-cooled mercury cadmium telluride detector. Two hundred fifty-six interferograms were coadded to generate a single spectrum, at nominal resolution of 8 cm⁻¹ with an encoding interval of 1 wavenumber/cm⁻¹. The spectra were then apodized with a triangular smoothing function before applying the Fourier transformation. In addition, band narrowing by the method of Fourier self-deconvolution was performed with smoothing parameters (k value) of 1.6 and 16 cm⁻¹ for band half-widths (14).

7AAD Staining and Flow Cytometry. Actinomycin D (7AAD, Calbiochem-Novabiochem, La Jolla, CA) was dissolved in acetone and diluted in PBS to a concentration of 200 μg/ml and was kept at −20°C, protected from light as described previously (15). Briefly, 100 μl of 7AAD solution were added to 10⁶ cells, suspended in 1 ml of PBS, and mixed well. WSU-CLL cells were stained for 20 min on ice while protected from light. Cells were pelleted, the supernatant was removed, and the pellet was washed twice with PBS. Samples from untreated and 24-h Bryo 1-treated cells were analyzed by flow cytometry. Scattergrams were generated by combining forward light scatter with 7AAD fluorescence.

WSU-CLL Xenografts. Four-week old female ICR-SCID mice were obtained from Taconic Laboratory (Germantown, NY). The mice were adapted and WSU-CLL xenografts were developed as described previously (11). Each mouse received 10⁷ WSU-CLL cells (in serum-free RPMI 1640) s.c. in each flank area. When s.c. tumors developed, the mice were sacrificed and the tumors were dissected and mechanically dis-
In Vitro Studies

**Effect of Bryo 1 and 2-CdA on Cell Growth.** Both Bryo 1 and 2-CdA showed a dose-dependent growth inhibitory effect on WSU-CLL cells. At a 10 nM concentration, Bryo 1-treated cells did not show any significant growth inhibition compared with untreated cells, except on day 4 (Fig. 1). At 100 nM, Bryo 1 showed some growth inhibition (on day 4) when compared with either control or 10 nM. A significant growth inhibition of WSU-CLL cells was noticed at 200 nM concentration. Bryo 1 at this concentration exhibited 63 and 39% inhibition compared with the control and 100 nM dose, respectively, on day 4. A 1000 nM concentration of Bryo 1 showed no significant advantage over the 200 nM concentration for growth inhibition and may not be clinically achievable.

On the other hand, 2-CdA at 11.2 μM showed a significant growth inhibition when compared with the control cells and cells treated with 5.6 μM, and exhibited 57 and 21.3% inhibition, respectively, on day 4. 2-CdA at concentrations of 16.8 and 22.4 μM was toxic (Fig. 2). Isobologram analysis to characterize the interaction between Bryo 1 and 2-CdA revealed that the combination of Bryo 1 (100 nM) with concentrations of either 5.6 or 11.2 μM of 2-CdA on day 4 was synergistic (data not shown).

To examine whether Bryo 1 can augment the inhibitory effect of 2-CdA, Bryo 1 at 100 nM and 2-CdA at 11.2 μM were chosen for the subsequent experiments. Of all of the different combination treatments tested, only the sequential addition of 100 nM Bryo 1 followed (72 h later) by 11.2 μM 2-CdA resulted in complete growth inhibition of WSU-CLL cells (Fig. 3). Adding 2-CdA 24 or 48 h after Bryo 1 showed less dramatic growth inhibition compared with its addition at 72 h. Reverse sequential additions (2-CdA, then Bryo 1) at different time points showed a lesser growth inhibiting effect on WSU-CLL cells as compared with the concurrent addition of Bryo 1 and 2-CdA (data not shown).

**Results**

**In Vitro Studies**

**Effect of Bryo 1 and 2-CdA on Cell Growth.** Both Bryo 1 and 2-CdA showed a dose-dependent growth inhibitory effect on WSU-CLL cells. At a 10 nM concentration, Bryo 1-treated cells did not show any significant growth inhibition compared with untreated cells, except on day 4 (Fig. 1). At 100 nM, Bryo 1 showed some growth inhibition (on day 4) when compared with either control or 10 nM. A significant growth inhibition of WSU-CLL cells was noticed at 200 nM concentration. Bryo 1 at this concentration exhibited 63 and 39% inhibition compared with the control and 100 nM dose, respectively, on day 4. A 1000 nM concentration of Bryo 1 showed no significant advantage over the 200 nM concentration for growth inhibition and may not be clinically achievable.

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3 Days in Culture

Fig. 3 Sequential addition of 2-CdA (Δ, 24 h; □, 48 h; ◦, 72 h) to Bryo 1-treated WSU-CLL cells in vitro (100 nM Bryo 1, 11.2 μM 2-CdA) compared with adding both agents at time zero (Bryo 1+2-CdA; ○).

Effect of Bryo 1 on AcP and TRAP. We examined untreated and Bryo 1-treated WSU-CLL cells for AcP and TRAP reactions. At 72 and 120 h, Bryo 1 was able to induce AcP and TRAP (positive reaction) in WSU-CLL cells (Table 1). The reaction was stronger after 120 h. The TRAP reaction is specific for HC leukemia (16, 17) and is typically negative in CLL.

Effect of Bryo 1 on Immunophenotypic Changes. We examined the expression of selected immunophenotypic markers (CD10, CD11c, CD22, and CD25) on untreated and Bryo 1-treated WSU-CLL cells at 24, 72, and 120 h. Untreated cells were highly positive for CD10 (99%), and negative for CD11c (2.8%), CD25 (1.3%), and CD22 (11.2%). Bryo 1 was able to induce CD11c (22.4%) and CD25 (13%) expression, and it up-regulated CD22 expression (92.4%) at 72 h (Fig. 4).

The coexpression of CD10/CD11c, CD10/CD25, CD22/CD11c, and CD22/CD25 in untreated control cultures (Fig. 4, top panel) was 5, 1.3, 2.8, and 1.1%, respectively, at 72 h. Treatment of the WSU-CLL cells with 200 nM of Bryo 1 for 72 h induced CD10/CD11c on 22% of the cells, CD10/CD25 on 13% of the cells, CD22/CD11c on 22.4%, and CD22/CD25 on 13% (Fig. 4, bottom panel).

The coexpression of CD22/CD11c on 22.4% of Bryo 1-treated WSU-CLL cells is unique to HC leukemia and monocytoïd B cell lymphoma (18–20). On the other hand, 13% of Bryo 1-treated cells coexpressed CD22/CD25, a specific expression also typically seen in HC leukemia (21–23).

Changes in Lipid and Protein Contents Observed in Bryo 1-treated WSU-CLL Cells by FT-IR Spectroscopy. A significant increase in the amount of lipids was found in WSU-CLL cells treated with Bryo 1 for 24 h compared with untreated control cells in all three replications. Fig. 5 (bottom panel) shows the two mean spectra of control and Bryo 1-treated cells in the range of 2800–3150 cm\(^{-1}\); this value is typical for C-H stretching vibrations [such as methyl (RCH\(_3\)), methylene (R\(_2\)CH\(_2\)), and methine (R\(_3\) CH) groups]. For the treated cells, this range indicates a massive change in protein contents. The difference spectra (top panel) in Fig. 5 demonstrates the existence of more lipids (filled arrows) and fewer proteins (open arrows) in the Bryo 1-treated cells indicated by two positive methylene bands (at 2853 and 2923 cm\(^{-1}\)) and negative methyl and amide B bands (at 2953 and 3060 cm\(^{-1}\)).

The spectral range between 800 and 1800 cm\(^{-1}\) (Fig. 6) provides absorbance readings which further indicate differences in lipid, protein, and now DNA content between 24-h Bryo 1-treated and untreated control cells. Fig. 6, bottom panel, depicts the spectroscopic difference between the 24-h Bryo 1-treated and untreated cells, which is then represented in the peaks and valleys of the top panel. Spectroscopically, the information here is based on a different methylene band at 1467 cm\(^{-1}\) and an ester band at 1740 cm\(^{-1}\) coming from the fatty acids in simple membrane lipids. Typical protein amide II and I features at 1550 and 1650 cm\(^{-1}\) indicate a relatively lower protein content. Arrows in the upper panel indicate an increased lipid and decreased protein content.

Decreased DNA Content. Our results demonstrated a significant decrease in DNA content when WSU-CLL cells were treated with Bryo 1 for 24 h. This can be seen by following the intensity of typical DNA bands. The IR spectra of WSU-CLL cells in Fig. 6 shows bands at 1000, 1100, and 1713 cm\(^{-1}\).

<table>
<thead>
<tr>
<th>Stain</th>
<th>Untreated WSU-CLL cells</th>
<th>Bryostatin-treated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72 h</td>
<td>120 h</td>
</tr>
<tr>
<td>AcP</td>
<td>−/+ “</td>
<td>+ +</td>
</tr>
<tr>
<td>TRAP</td>
<td>−</td>
<td>+ +</td>
</tr>
</tbody>
</table>

“−”, negative; −/+ , equivocal; +, mild; +++, moderate; ++++, strong positivity.

Table 1 Bryo 1-induced enzymatic changes in WSU-CLL cells in vitro
Differences between Bryo 1-treated and untreated WSU-CLL cells revealed a decrease in the DNA content of the 24 h Bryo 1-treated cells at all of these bands.

**Induction of Apoptosis in WSU-CLL Cells by Bryo 1.**

Typical fluorescence-activated cell sorting scattergrams of control and Bryo 1-treated cells are shown in Fig. 7 (left and right panels). This shows the three regions defined by 7AAD staining: 7AAD-negative (R3-live cells), 7AAD-dim (R2-apoptotic cells), and 7AAD-bright (R1-late apoptotic or dead cells). As shown in Fig. 7, Bryo 1 was able to induce apoptosis on 16% of WSU-CLL cells at 24 h. Bryo 1 increased late apoptotic + apoptotic from 8% + 8% in the control to 24% + 16% after 24 h.

**In Vivo Studies**

Table 2 shows the efficacy preclinical trial design for Bryo 1, 2-CdA, and their combinations in one and two cycles. Briefly, Bryo 1 was given alone, concurrently with 2-CdA, and before or after 2-CdA. Bryo 1 was injected i.p. and was used at its MTD (12), and 2-CdA was injected s.c. at its MTD, which was determined in this study to be 35 mg/kg/day. When 2-CdA was given in combination or sequentially with Bryo 1 (75 µg/kg), the MTD for 2-CdA in mice remained 35 mg/kg/day. On the basis of the MTD of 2-CdA, we used a dose of 30 mg/kg/day in subsequent studies (Table 2).

Table 3 shows antitumor activity of Bryo 1, 2-CdA, and their combinations against WSU-CLL-SCID mice xenografts. Activity is illustrated by T/C (%), T/C kill values were 11%, 16 days, and 1.10 for the one-cycle schedule and 12%, 20 days, and 1.36 for the two-cycle schedule. The reverse sequential addition of Bryo 1 and 2-CdA, or the concurrent use of both agents, did not show any activity against WSU-CLL-bearing mice. Administering Bryo 1 for 3 days followed by 2-CdA for 5 days (starting 4 days later) did not show any antitumor activity. The median tumor weight of WSU-CLL SCID xenografts in all treatments and control were studied. Clearly, the results indicate that 2-CdA given after Bryo 1 for either one (X1) or two cycles (X2) was more effective in extending the duration for the tumors to reach 2000 mg, compared with other treatments (Fig. 8). Mean and range of tumor weight in mg, as well as P values, were calculated (Table 3). Results showed that there were significant differences between the control group and the groups with Bryo 1 (alone) or Bryo 1 followed by 2-CdA (either X1 or X2). The P values were 0.05, 0.03, and 0.03, respectively. On the other hand, no significant differences were observed between the group given 2-CdA alone, the group given Bryo 1 + 2CdA, the group given Bryo 1 for 3 days followed by 2-CdA for 5 days, and control group.

**DISCUSSION**

We have previously reported that Bryo 1 induces differentiation of CLLs in vitro to a HC stage (5). This study tested the hypothesis that Bryo 1-differentiated CLL cells are more susceptible to 2-CdA, a drug active in treating de novo HC leukemia but not active in fludarabine-resistant CLL (24-26). A recently established EBV-negative CLL line (WSU-CLL) from a patient resistant to chemotherapy including fludarabine was used to test this hypothesis. Here, we demonstrate for the first time that Bryo 1 given to SCID mice bearing CLL tumors showed reasonable antitumor activity. Moreover, administering 2-CdA after Bryo 1 resulted in improved antitumor activity. This sequential interaction between Bryo 1 and 2-CdA is se-
Bryo 1 received considerable attention in the past few years as an antineoplastic agent (3–5). In addition to its antitumor activity, Bryo 1 has been found to possess powerful differentiating properties on B-cell tumors (20, 27). In vitro, our results indicate that Bryo 1 and 2-CdA show a dose-dependent growth inhibitory effect on WSU-CLL cells (Figs. 1 and 2). The use of Bryo 1 (100 nm) followed 72 h later by 2-CdA (11.2 μM) resulted in complete growth inhibition of WSU-CLL cells (Fig. 3).

Cells in the HC stage usually express specific enzymes and markers. Bryo 1 was able to induce AcP and TRAP (positive reaction) in WSU-CLL cells (Table 1). The TRAP reaction is specific for HC leukemia (16, 17) and is typically negative in CLL. Bryo 1 also induced the expression of specific phenotype HC markers, such as CD11c and CD25 (Fig. 4). More importantly, Bryo 1-treated cells coexpressed CD22/CD11c and CD22/CD25, which is also typically seen in HC leukemia (18, 20–23). This is consistent with our previous findings that Bryo 1 induces morphological, enzymatic, and phenotypic features of HC leukemia on fresh CLL cells taken from the peripheral blood of patients with this disease (5). Drexler et al. (3) reported that Bryo 1 was capable of inducing differentiation of B-CLL cells (3). Subsequently, we were able to demonstrate that Bryo 1 induces modulation of the human non-Hodgkin’s B-lymphoma cell lines (20) and acute lymphoblastic leukemia cell line Reh (27).

FT-IR spectroscopy has become a powerful tool in diagnosis of CLL; it is used to study cellular components (DNA, proteins, and lipids) of purified CLL cells from patients in comparison with normal lymphocytes (9). CLL cells usually have higher DNA and protein content and a lower lipid content than normal peripheral blood mononuclear cells. More recently, this technique has been extended to evaluation of the prognosis of CLL by studying the variations observed in the FT-IR spectra of CLL cells to predict the ex vivo sensitivity/resistance to treatment with two common antileukemic agents, 2-CdA and chlorambucil (10). Here we show that treatment with Bryo 1 resulted in a higher lipid content and lower protein and DNA content dependent because neither concurrent nor reverse sequential use of the two agents showed activity.

Fig. 5  The difference in spectra demonstrates the existence of more lipids (filled arrows) and fewer proteins (open arrows) in the Bryo 1-treated cells, indicated by two positive methylene bands (at 2853 and 2923 cm⁻¹) and negative methyl and amide B bands (at 2953 and 3060 cm⁻¹).
Fig. 6  IR spectra of untreated and Bryo 1-treated WSU-CLL cells shows bands specific for DNA at 1000, 1100, and 1713 cm\(^{-1}\) (bottom panel). These bands showed reduced intensity after 24 h of Bryo 1 treatment (data represent average of three experiments). This reduced intensity is the difference between Bryo 1-treated and control cells (top panel).

Fig. 7  Scattergrams of 7AAD-stained cells are shown. Left, untreated WSU-CLL cells; right, WSU-CLL cells treated with 200 nm concentration of Bryo 1 for 24 h. R1, late apoptotic or dead cells; R2, apoptotic cells; R3, live cells; FSC, forward light scatter; FL3, 7AAD fluorescence.

contents (Figs. 5 and 6). Because lipids are located in the cell membrane, these findings suggest that there are changes in membrane structure and/or in the whole composition of the CLL cells, which may explain the morphological changes (e.g., larger size) and cytoplasmic extensions seen in WSU-CLL cells treated with Bryo 1. Previously, our group found that the alteration of membrane lipids plays an important role in the resistance of CLL to chlorambucil and to the nucleoside analogues
Table 2  *In vivo* Bryo 1 and 2-CdA dose and schedule for WSU-CLL-bearing SCID mice

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose</th>
<th>Route</th>
<th>No. of animals</th>
<th>No. of injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent (control)</td>
<td>0.0 mg/kg/inj/day</td>
<td>i.p.</td>
<td>6†</td>
<td>20</td>
</tr>
<tr>
<td>Bryostatin 1</td>
<td>75 μg/kg/inj/day</td>
<td>i.p.</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>2-CdA</td>
<td>30 mg/kg/inj/day</td>
<td>s.c.</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Bryo 1 + 2-CdA (2 cycles)</td>
<td>75 μg/kg/inj/day</td>
<td>i.p.</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>Bryo→2-CdA (1 cycle)</td>
<td></td>
<td></td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Bryo→2-CdA (2 cycles)</td>
<td></td>
<td></td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>2-CdA→Bryo (2 cycles)</td>
<td></td>
<td></td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>Bryo→2-CdA (3 + 5)‡</td>
<td></td>
<td></td>
<td>6</td>
<td>8</td>
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</tbody>
</table>

† 1 cycle (for one agent), treatment for 2 weeks, 5 days a week. 1 cycle (for two agents), treatment for 2 weeks (first agent followed by second agent), 5 days a week. 2 cycles, treatment for 4 weeks (first agent followed by second agent), 5 days a week.

Table 3  *In vivo* activity of Bryo 1 and 2-CdA against WSU-CLL-SCID model

<table>
<thead>
<tr>
<th>Agent</th>
<th>T/C (%)</th>
<th>T − C (days)</th>
<th>Log₁₀ kill (gross)</th>
<th>Mean (mg)</th>
<th>Range</th>
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<tr>
<td>Diluent (control)</td>
<td>100</td>
<td>0.00</td>
<td>0.00</td>
<td>1557.0</td>
<td>(3376–836 mg)</td>
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<tr>
<td>Bryo 1</td>
<td>19</td>
<td>13</td>
<td>0.90</td>
<td>400.7*</td>
<td>(1626–14 mg)</td>
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<tr>
<td>2-CdA</td>
<td>100</td>
<td>0.00</td>
<td>0.48</td>
<td>586.5 NS</td>
<td>(1372–14 mg)</td>
</tr>
<tr>
<td>Bryo + 2CdA (2×)</td>
<td>60</td>
<td>7</td>
<td>1.10</td>
<td>243.3*</td>
<td>(726–14 mg)</td>
</tr>
<tr>
<td>Bryo→2CdA (1×)</td>
<td>11</td>
<td>16</td>
<td>0.48</td>
<td>243.3*</td>
<td>(726–14 mg)</td>
</tr>
<tr>
<td>Bryo→2CdA (2×)</td>
<td>12</td>
<td>20</td>
<td>0.27</td>
<td>509.2 NS</td>
<td>(1099–14 mg)</td>
</tr>
<tr>
<td>2CdA→Bryo (2×)</td>
<td>67</td>
<td>4</td>
<td>0.00</td>
<td>1119.4 NS</td>
<td>(2745–14 mg)</td>
</tr>
</tbody>
</table>

* Significant at *P* = 0.05.

‡ NS, not significant at *P* = 0.05.

Highly significant at *P* = 0.05.

Three days of Bryo 1, followed by 2-CdA 4 days later.

Fig. 8  Median tumor weight in mg of WSU-CLL SCID xenografts in various treatments. ○, control; ▲, 2-CdA→Bryo (×2); □, Bryo+2-CdA (×2); △, Bryo→2-CdA (×1); ●, Bryo→2-CdA (×2).

fludarabine and 2-CdA (10). The bands at 1000, 1100, and 1713 cm⁻¹ in the spectra indicate a lower DNA content in Bryo 1-treated cells. A possible explanation for this reduction of DNA can be an enhanced metabolism (increase in vesicular bodies) or even induction of programmed cell death subsequent to Bryo 1 treatment. To determine whether the cells underwent apoptosis or simply showed necrosis, WSU-CLL cells were treated with Bryo 1 for 24, 48, and 72 h. The results of 7AAD staining and flow cytometric analysis indicate that Bryo 1 induced apoptosis within 72 h (Fig. 7).

The ability of Bryo 1 to differentiate CLL cells to HC stage (morphological, enzymatic, and phenotypic), and to change basic cellular contents (increase lipid and decrease protein and DNA content) similar to that seen in CLL sensitive to 2-CdA, is unique and could form the basis for new strategies to treat CLL. Bryo 1 can be used in conjunction with 2-CdA, which is very active against HC leukemia. To test this hypothesis *in vivo*, a series of different schedules and combinations using Bryo 1 and 2-CdA against the WSU-CLL model were conducted (Table 2). Results showed that WSU-CLL was resistant to 2-CdA but not to Bryo 1. Interestingly, SCID mice bearing WSU-CLL tumors treated with Bryo 1 followed by 2-CdA showed the highest antitumor activity compared with other Bryo 1 and 2-CdA combinations (Table 3). The tumor growth inhibition ratio (*T/C*), the tumor growth delay (*T−C*) and the log₁₀ kill in Bryo 1 followed by 2-CdA treatment were 11%, 16 days, 1.10 after one cycle and 12%, 20 days, and 1.36 after two cycles. Statistical analysis revealed that there was significant tumor weight decrease when Bryo 1 was given alone (*P* = 0.05) and when Bryo 1 was followed by 2-CdA for one (*P* = 0.03) and two (*P* = 0.03) cycles, compared with the untreated control group. Fig. 7 shows the median tumor weight of WSU-CLL in SCID we-
nografts. Conversely, administering 2-CdA before Bryo 1 or concurrently with Bryo 1 showed no antitumor activity. It is worth noting that administering Bryo 1 for only 3 days, followed by 2-CdA starting 4 days later, did not show any antitumor activity (Table 3). There are two possible explanations for this finding: (a) Bryo 1 therapy for 3 days is not sufficient to induce the optimum differentiation in vivo allowing increased susceptibility to 2-CdA (this possibility is supported by our observation that tumors began to grow during the 4-day hiatus between completion of Bryo 1 treatment and beginning of the 2-CdA); or (b) this latter observation also suggests that 2-CdA has to be given immediately following Bryo 1 for full benefit. The 4-day hiatus was based on scheduling convenience; drugs were given at the beginning of the week.

It had been reported that the deoxycytidine kinase levels were significantly higher and the 5'-nucleotidase levels were significantly lower in CLL cells of patients who responded to 2-CdA than in nonresponders (28). The levels of these two enzymes in Bryo 1-treated WSU-CLL cells and control cells were studied. Our results showed that the deoxycytidine kinase: 5'-nucleotidase ratio (mean of three replications) was higher in Bryo 1-treated cells at 12 h compared with controls (data not shown). In summary, these in vitro and in vivo studies encourage the evaluation of Bryo 1 and 2-CdA sequential regimens in carefully designed clinical trials against CLL.

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Sequential treatment of human chronic lymphocytic leukemia with bryostatin 1 followed by 2-chlorodeoxyadenosine: preclinical studies.

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