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The *bcl*-2 and p53 oncoproteins can be modulated by bryostatin 1 and dolastatins in human diffuse large cell lymphoma

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The effects of dolastatin 10 (Dol10) and dolastatin 15 (Dol15) alone, and after treatment with bryostatin 1 (Bryo1), on human diffuse large cell lymphoma cell line (WSU-DLCL2) were studied. At a concentration of 1.0 ng/ml Dol10 and Dol15 showed significant growth inhibition ($p < 0.05$). This inhibition was intensified when the cells were pretreated for 24 h with 200 nM Bryo1. Bryo1, Dol10 and Dol15 induced apoptosis which was seen on morphological examination, by flow cytometry and DNA fragmentation on agarose gel electrophoresis. Cells pretreated with Bryo1 and then exposed to Dol10 showed an increase in apoptosis compared with cells that were treated with the Dol10, Dol15 alone. Immunocytochemistry revealed that WSU-DLCL2 cells express the *bcl*-2 oncoprotein constitutively. *bcl*-2 expression was decreased when cells were treated with Bryo1, Dol10 or Dol15 and abolished with the Bryo1/Dol10 combination. WSU-DLCL2 cells were negative for p53 protein expression, upon treatment with Bryo1 or Dol10, the expression of p53 was weak and moderate with the Bryo1/Dol10 combination. The inverse correlation between *bcl*-2 and p53 oncoprotein expression seems to be related to induction of apoptosis in this lymphoma cell line.

Key words: B cell, *bcl*-2, bryostatin 1, diffuse large cell lymphoma, p53, dolastatin 10, dolastatin 15.

Introduction

The diffuse large cell non-Hodgkin's lymphoma (DLCL) has been classified as an intermediate grade lymphoma according to the International Working Formulation.[1] Using standard chemotherapy with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone), 30–40% of patients achieve long-term survival.[2] Other regimens have been tried but were not found to increase the survival rate when compared with the original CHOP regimen.[3,4] It is important that regimens incorporating newer, more potent antineoplastic agents be devised to increase the existing rate of cure. A combination of cytotoxic and biologic agents having a different mode of action can be one approach to possibly achieving that goal.[5]

In the past few years there has been an ongoing search for newer anticancer agents derived from natural plant or animal sources. These efforts have revealed several potentially new useful compounds including bryostatin 1 (Bryo1),[6,7] dolastatins[8,9] and the halichondrins.[10] The dolastatins are small peptides isolated from the sea hare *Dolabella auricularia*. They are powerful antineoplastic compounds that cause cells to arrest in metaphase by binding to microtubule components in the cell, particularly to tubulin.[11] Bryo1, a macrocyclic lactone isolated from the marine *Bugula neritina*, is a potent activator of protein kinase C.[6,12] It has antitumor, immune modulating and differentiating capacity on a number of B cell lymphomas and leukemias.[12–14]

Recently we have demonstrated that Bryo1 given 24 h before vincristine (VCR) significantly increased the antitumor activity against Waldenstrom's macroglobulinemia xenograft compared with VCR alone.[5] The dolastatins are more effective in inhibiting tubulin polymerization at lower doses as compared with VCR[15] and have additional mechanisms of action on tubulin which are quite different from those caused by vinca alkaloids.[11,16,17] Therefore, it is crucial to study their effects, alone and in combination with Bryo1.

Materials and methods

Cell culture

The malignant human cell line WSU-DLCL2 (diffuse large cell lymphoma) was established in our labora-

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tory and used in this study.[18] The cell line grows in liquid culture consisting of RPMI 1640 supplemented with 1% glutamine (Gibco BRL, Gaithersburg, MD), 10% (v/v) heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were seeded at a concentration of 0.8×10^6/ml in a 24-well plate and incubated at 37°C in a 5% CO_2 humidified incubator after adding drugs in the concentrations described below.

Drugs and reagents

Bryo1 was dissolved in dimethylsulfoxide (DMSO) at 10^{-3} M, further diluted with PBS and then used in a final concentration of 200 nM. This concentration was found to be optimum in previous study.[14] Dol10 and Dol15 were also dissolved in DMSO, and were used in a pilot experiment at concentrations of 0.01, 0.1 and 1.0 ng/ml. Dolastatins were used at a concentration of 1 ng/ml throughout this study due to their activity at this concentration.

Growth analysis

WSU-DLCL2 cells were seeded in a 24-well culture plate (Costar, Cambridge, MA) at a concentration of 0.8×10^6 viable cells/ml. Untreated (control), Bryo1 (200 nM), Dol10 (1 ng/ml), Dol15 (1 ng/ml), Bryo1/Dol10 and Bryo1/Dol15 treated cultures were set in four replications. Cell count and viability were then obtained daily using Trypan blue (0.4%) exclusion (Gibco, Grand Island, NY).

Analysis of apoptosis

Morphological studies. Cytocentrifuge smears from all cultures were prepared using the Cytospin 2 centrifuge (Shandon Southern Instruments, Sewickley, PA) daily for 4 days. The smears were air dried, stained with tetrachrome for 5 min and analyzed under a light microscope. Five different high power fields were counted for viable, mitotic, apoptotic and dead cells. At least 300 cells were counted and statistically analyzed. Features of apoptosis that were looked for included cell shrinkage, nuclear chromatin condensation, formation of membrane blebs and apoptotic bodies.

Flow cytometric detection of apoptosis. Flow cytometric detection of apoptosis was conducted 48 h after the addition of Bryo1. One million cells were suspended in PBS and then fixed in absolute alcohol for 30 min at 4°C. After centrifugation, cells were resuspended in PBS followed by the addition of a buffer containing disodium phosphate and citric acid, and incubated at room temperature for 5 min. Cells were then treated with RNase for 40 min at 37°C, stained with propidium iodide and analyzed by flow cytometry (FCM) on a FACScan as routinely done at our laboratory. Apoptotic cells show on the DNA histogram as a peak in the hypodiploid region.

Analysis of DNA fragmentation by agarose gel electrophoresis. DNA was extracted from cells treated with Bryo1, Dol10, Dol15, Bryo1/Dol10, Bryo1/Dol15 and control cultures using 10 mM Tris-HCl/1 mM EDTA, pH 8.0 (TE), containing 0.2% Triton X-100. Extraction was conducted 48 h after adding Bryo1. DNA was precipitated from the lysate by 0.1 volumes of 5 M Sodium acetate (pH 5.0) and 3 volumes of 95% ethanol. After centrifugation, DNA pellets were air dried and resuspended in 25 μl of TE containing 0.1 SDS. Loading buffer was added to the samples at 1:5 (v/v) ratio and the samples were incubated for 10 min at 60°C, followed by agarose gel (0.75%) electrophoresis in Tris borate buffer (45 mM Tris borate/1 mM EDTA, pH 8.0). DNA was visualized with ethidium bromide under UV light and photographed.

Analysis of bcl-2 *and p53 oncoprotein expression by immunocytochemistry.* Cytocentrifuge smears on round coverslips were prepared using the Cytospin 2 centrifuge from control, Bryo1, Dol10, Dol15, Bryo1/Dol10 and Bryo1/Dol15 treated WSU-DLCL2 cells. The Smears were obtained 48 h after adding Bryo1. Cells were fixed with 95% ethanol and 5% glacial acetic acid for 30 min at room temperature. Cells were then washed with 0.1 M PBS and incubated overnight with mouse monoclonal anti-human *bcl*-2 (Dako, CA) and mouse anti-human p53 antibodies (Oncogenes Science, New York, NY) at dilutions of 1:60 and 1:25, respectively. The cells were washed extensively with PBS and incubated for 2 h with goat anti-mouse antibodies conjugated to alkaline phosphatase. A subsequent enzyme catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT) was used to visualize the protein by inducing a blue precipitate.

Results

Growth analysis

The effects of Bryo1, Dol10, Dol15 and their combination on the growth of WSU-DLCL2 cells are

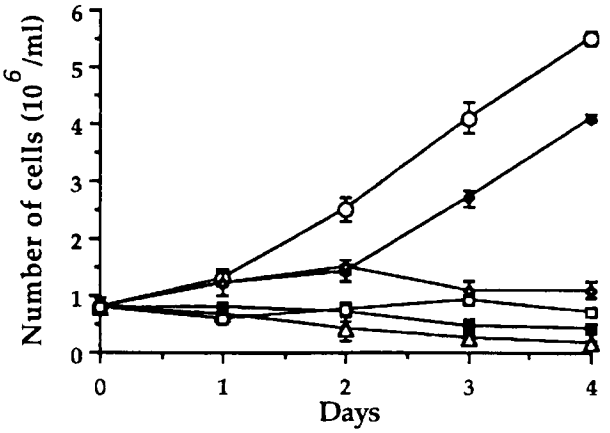

Figure 1. Effects of control (○), Bryo1 (◆), Dol10 (■), Dol15 (◆), Bryo1/Dol10 (△) and Bryo1/Dol15 (□) on cell growth of WSU-DLCL2.

shown in Figure 1. Bryo1 caused moderate growth inhibition. Both Dol10 and Dol15 caused significant growth inhibition with Dol10 being more potent than Dol15 ($p < 0.05$). Adding Bryo1 24 h before Dol10 or Dol15 also caused significant inhibition with the Bryo1/Dol10 combination being more toxic to the cells than the Bryo1/Dol15 combination. The Bryo1/Dol10 combination caused complete growth arrest during the first 24 h with no signs of recovery thereafter.

Apoptosis Analysis

Morphological studies were conducted on all treatments to assess for viability, mitosis, apoptosis and death. Cells treated with Bryo1, Dol10 and Dol15 showed characteristic features of apoptosis like cell shrinkage, nuclear condensation and apoptotic bodies (Figure 2). Features of cell death were determined as a function of cell swelling, nuclear expansion and cytolysis. As shown in Figure 3, Dol10 and Dol15 caused mitotic arrest in 13.3 and 8.0% of cells, respectively, and this was not significantly altered by pretreatment with Bryo1. Bryo1 induced apoptosis in 8.1% of cells, Dol10 in 8.9% and Dol15 in 4.0% as compared with the control which showed no apoptosis ($p < 0.05$) (Figure 3). The combination of Bryo1 with Dol10 or Dol15 showed apoptosis in 13.8 and 7.9% of cells, respectively ($p < 0.05$). Dol10 caused death in 11.1% of cells, Dol15 in 5.5% and Bryo1 in 1.6% of cells. The Bryo1/Dol10 and Bryo1/Dol15 combinations caused death in 14.9 and 9.2% of cells, respectively (Figure 3).

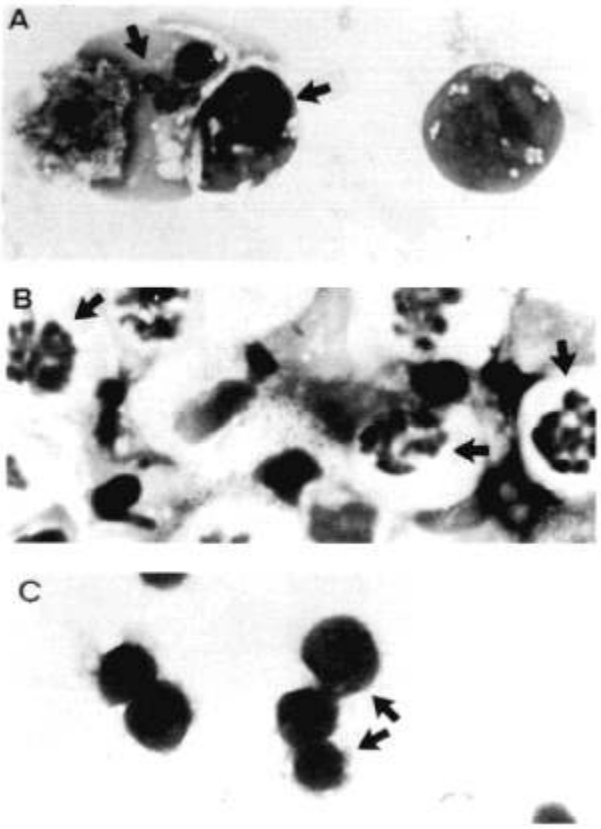

Figure 2. Photograph showing the characteristic features of treated WSU-DLCL2 cells (×840). (a) Cells treated with Bryo1 (200 nM) showing apoptosis, note the nuclear condensation and apoptotic bodies, characteristic features of apoptosis. On the right side, a normal looking cell (control) is seen, (b) Cells treated with Dol10 (1 ng/ml) showing mitosis and (c) cells treated with the Bryo1/Dol10 combination showing cell death.

FCM was used to verify the morphological observation for apoptotic cells. Apoptosis was detected in 9.5, 9.1 and 8.2% of the cells treated with Bryo1, Dol10 and Dol15, respectively (Table 1). The combination of Dol10 or Dol15 with Bryo1 caused an increase in apoptosis as compared with a single agent (14.5 and 10.1, respectively). Bryo1/Dol10 was statistically different compared with other treatments ($p < 0.05$).

Apoptosis is characterized by the fragmentation of the DNA to nucleosomes. Generally eukaryotic cells die by necrosis, in which DNA remains intact. Thus, to confirm that the death of the cells was caused by the apoptosis pathway, an electrophoresis analysis was conducted on treated and control cells (Figure 4). A classical laddering pattern resulting from the generation of multiples of nucleosome

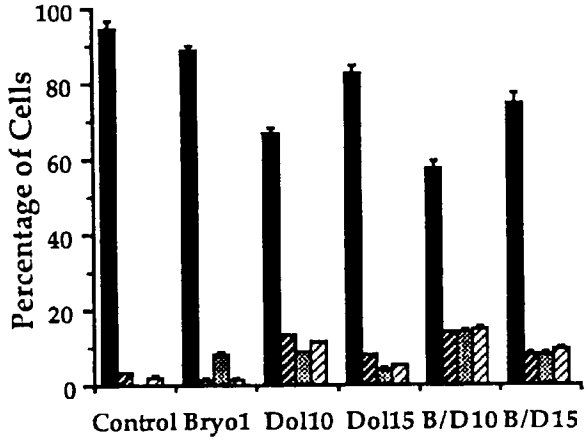

Figure 3. Bar chart showing the percentage of viable (■), mitotic (▨), apoptotic (□) and dead (⊡) cells in control, Dol10, Dol15 and Bryo1/Dol10 and Bryo1/Dol15 combination treated WSU-DLCL2 cells.

Table 1. FCM analysis, after 48 h, showing the percentage of cells in various phases of the cell cycle in control and treated WSU-DLCL2 cells

Treatment	Apoptosis	G_0/G_1	S	G_2M
Control	0.0	62.5	30.8	6.7
Bryo1	9.5	61.7	22.7	6.1
Dol10	9.1	49.6	26.2	15.1
Dol15	8.2	53.5	22.1	16.2
Bryo1[a]/Dol10	14.5[b]	40.3	30.0	15.2
Bryo1/Dol15	10.1	57.8	19.3	12.8

[a] Bryo1 was added 24 h prior to Dol10 and Dol15.
[b] Significant at $p < 0.05$

fragments was seen in all treated cultures, indicating that these cells underwent apoptosis. The apoptosis seen with DNA fragmentation is similar to what was seen using flow cytometry or morphological techniques.

bcl-2 and p53 oncoproteins expression

The relative expressions of the oncoprotein *bcl*-2 and the tumor suppressor protein p53 were examined by immunocytochemistry. The untreated (control) cells strongly expressed the *bcl*-2 oncoprotein (Table 2). However, *bcl*-2 expression was relatively weak in cells treated with all agents used in this study and it was completely abolished with the Bryo1 Dol10 combination. No p53 expression was observed in the control WSU-DLCL2 cells, while it was weak in cells subjected to Bryo1 and Dol10 alone. However, the expression was up-regulated for the Bryo1 Dol10 combination (Table 2).

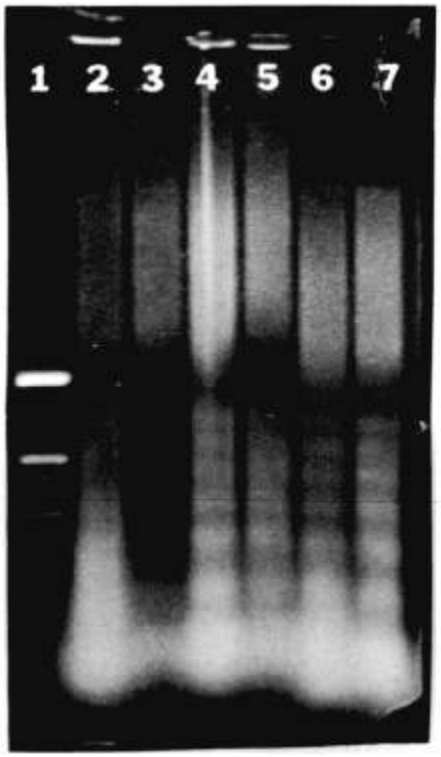

Figure 4. Classical laddering pattern caused by DNA fragmentation on agarose gel electrophoresis induced by the drugs. 1, DNA mass ladder supplied by Gibco BRL (range 100–800 bp); 2, control; 3, Dol15; 4, Bryo1/Dol10; 5, Dol10; 6, Bryo1; 7, Bryo1/Dol15.

Table 2. Immunocytochemistry studies for *bcl*-2 and p53 oncoproteins expression on treated and control WSU-DLCL2 cells

Agent	*bcl*-2	p53
Control	+ + +	−
Bryo1	+	+
Dol10	+	+
Dol15	+ +	−
Bryo1/Dol10	−	+ +
Bryo1/Dol15	+	−

−, negative; +, weak; + +, moderate; + + +, strong expression

Discussion

Bryo1 has been shown to have antineoplastic[7] and antileukemic[19] properties combined with stimulatory actions on normal hematopoietic progenitors[20]. The mechanism by which Bryo1 exerts its bi-directional action is not known at the present time. The Dolastatins have been shown to be inhibitory to leukemic cells by arresting them in metaphase.[11]

Thus, Bryo1 and the dolastatins appear to possess different mechanisms of action which may be complementary to each other and could be exploited clinically.

Bryo1, individually, caused a significant growth arrest in the first 48 h after which growth recovery was observed. However, this growth inhibition can be augmented and prolonged in the presence of the dolastatins, mainly Dol10 (Figure 1). Growth inhibition of WSU-DLCL2 was correlated with development of apoptosis (Figures 2–4). The apoptotic bodies were seen in all treated cells, but it was intensified with the Bryo/Dol10 combination. p53 and *bcl*-2 are the primary molecular mediators of cancer related apoptosis[21]. Exposing WSU-DLCL2 cells to Bryo1, Dol10, Dol15 or their combinations reduced *bcl*-2 expression and up-regulated p53 expression (Table 2). On the other hand, the Bryo1/Dol10 combination abolished completely *bcl*-2 and activating p53 expression. It has been reported that over-expression of p53 can induce apoptosis.[22] *bcl*-2, on the other hand, activated by chromosomal translocation in follicular lymphoma, demonstrated a profound capacity to block apoptosis.[23] Thus, *bcl*-2 and the factors with which it interacts, probably p53, likely comprise a common pathway for apoptosis. However, additional pathways might exist as well.

Recently,[5] we have demonstrated that the combination VCR with Bryo1 significantly increased the antitumor activity of Bryo1 in the Waldenstrom macroglobulinemia xenograft model. Dol10, at low dose concentrations, had more growth inhibitory effect on WSU-DLCL2 compared with VCR (data not shown). This might be due to its better effectiveness in inhibiting tubulin polymerization than the clinically employed cytotoxic agent VCR.[15] Bryo1 given 24 h before Dol10 increased growth inhibition, abolished *bcl*-2 expression, expressed p53 and induced apoptosis in WSU-DLCL2 cells. In conclusion, Dol10 has shown to be a promising antineoplastic agent which will be expected to enter phase I clinical trials shortly.

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