

# Phase I and Correlative Study of Combination Bryostatin 1 and Vincristine in Relapsed B-Cell Malignancies

Afshin Dowlati,<sup>1</sup> Hillard M. Lazarus,<sup>1</sup>  
Paul Hartman,<sup>1</sup> James W. Jacobberger,<sup>1</sup>  
Cecilia Whitacre,<sup>1</sup> Stanton L. Gerson,<sup>1</sup>  
Pamela Ksenich,<sup>1</sup> Brenda W. Cooper,<sup>1</sup>  
Phyllis S. Frisa,<sup>1</sup> Megan Gottlieb,<sup>1</sup>  
Anthony J. Murgo,<sup>2</sup> and Scot C. Remick<sup>1</sup>

<sup>1</sup>Developmental Therapeutics Program and the Division of Hematology/Oncology, Department of Medicine, Case Western Reserve University School of Medicine and University Hospitals of Cleveland, Cleveland, Ohio, and <sup>2</sup>National Cancer Institute, Bethesda, Maryland

## ABSTRACT

**Purpose:** Bryostatin 1 activates protein kinase C (PKC) with short-term exposure and results in depletion of PKC with prolonged exposure. Preclinical *in vitro* and *in vivo* studies demonstrate synergistic activity and increased tumor apoptosis in B-cell malignancies when a prolonged infusion of bryostatin 1 is followed by vincristine.

**Experimental Design:** We embarked on a Phase I trial of bryostatin 1 as a 24-h continuous infusion followed by bolus vincristine in patients with refractory B-cell malignancies other than acute leukemias. Twenty-four evaluable patients were enrolled.

**Results:** The dose-limiting toxicity was myalgia. The MTD and recommended Phase II dose of bryostatin 1 was 50  $\mu\text{g}/\text{m}^2/24$  h followed by vincristine 1.4  $\text{mg}/\text{m}^2$  (maximum total dose of 2 mg) repeated every 2 weeks. Significant antitumor activity was observed in this relapsed population, including patients who had failed high-dose chemotherapy. This included 5 durable complete and partial responses and 5 patients with stable disease lasting  $\geq 6$  months (range, 6–48+ months). Median time to response was 8 months. Correlative studies demonstrated a progressive increase in serum interleukin-6 with bryostatin 1 infusion followed by an additional increase after vincristine. Flow cytometry for

detection of apoptosis in B and T cells showed an initial decrease in apoptotic frequency in CD5+ cells within 6 h of bryostatin 1 infusion compatible with its known increase in PKC activity in the majority of patients followed by a return to baseline or overall increase in apoptotic frequency after completion of infusion. All (5 of 5) patients who had an overall increase in apoptotic frequency in CD5+ cells achieved either a clinical response or prolonged stable disease. Four of these 5 patients did not have the initial decrease in apoptosis at 6 h.

**Conclusions:** Given the lack of myelosuppression and early evidence of clinical efficacy, additional exploration of this regimen in non-Hodgkin's lymphoma and multiple myeloma is warranted.

## INTRODUCTION

Bryostatin 1 is one of the family of >13 compounds with a multiringed macrocyclic lactone structure isolated from the marine bryozoan *Bugula neritina* in 1982 by Pettit *et al.* (1) as part of the NCI<sup>3</sup> natural products program. The wide range of biological activities of bryostatin 1 include PKC modulation, hematopoietic and immune stimulation, and induction of differentiation of both myeloid and lymphoid cell lines. As a single agent, bryostatin exerts antiproliferative effects on a number of cell lines at concentrations ranging from <1.0 to 200 nm. *In vitro* antiproliferative effects have been observed against human B-cell lymphoma cell lines (2), human myeloid and lymphoid leukemias (3), as well as various solid tumor cell lines (4, 5). Single-agent activity has also been demonstrated *in vivo* against murine P388 leukemia system (1), L10A B-cell lymphoma, and B16 melanoma (2). It has been reported that continuous exposure to bryostatin 1 is required to maximize the drug's antiproliferative effect *in vitro* against leukemias and lymphomas (3).

Although the exact mechanism of action of bryostatin 1 is unknown, it has been shown *in vitro* to activate PKC activity with short-term exposure by inducing translocation of the protein from its cytoplasmic to its membrane-associated active fraction (6). Conversely, prolonged exposure results in membrane depletion of PKC and results in inactivation of the PKC activity as a consequence of proteosomal degradation after ubiquitination (7). Similar degradation effects have been seen on bcl-2 (8). Previous studies have suggested that PKC inhibitors

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**Requests for reprints:** Afshin Dowlati, Division of Hematology/Oncology, University Hospitals of Cleveland, 11100 Euclid Avenue, Cleveland, OH 44106. Phone: (216) 844-5181; Fax: (216) 844-5234; E-mail: axd44@cwru.edu.

<sup>3</sup> The abbreviations used are: NCI, National Cancer Institute; PKC, protein kinase C; mdr, multidrug resistance; DLT, dose-limiting toxicity; MTD, maximum-tolerated dose; PARP, poly(ADP-ribose) polymerase; IL, interleukin; IL-2R, IL-2 soluble receptor; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin's lymphoma; MM, multiple myeloma; PET, positron emission tomography; BM, bone marrow; PBMC, peripheral blood mononuclear cell; DLCL, diffuse large cell lymphoma; CHOP, cyclophosphamide-Adriamycin-vincristine-prednisone; EOI, end of infusion.

Table 1 Bryostatins/Vincristine dose escalation schema

Dose level	Dose		No. of Patients			
	Bryostatins ( $\mu\text{g}/\text{m}^2/\text{day}$ )	Vincristine ( $\text{mg}/\text{m}^2$ ) (maximum total dose = 2 mg)	New	De-escalated to level (for Bryostatins only)	Total	No. of courses (7/03)
1	12.5	1.0	3		3	13
2	16.5	1.4	3		3	16
3	22.0	1.4	3		3	6
4	30.0	1.4	3		3	50
5	40.0	1.4	3	2	5	34
6	50.0	1.4	6	3	9	56
7	62.5	1.4	4		4	7
Total			25			182

may have antineoplastic activity by mechanisms other than PKC inhibition, including immunomodulatory effects (9).

Pretreatment with bryostatins 1 for 24 h followed by vincristine *in vitro* enhanced apoptosis in a human diffuse large cell lymphoma cell line (WSU-DLCL2) compared with either drug alone (10). WSU-DLCL2 is a mature B-cell line that expresses the *mdr* phenotype. This increased apoptosis was associated with down-regulation of *bcl-2* and increased expression of the *p53* protein. In a later study, Al-Katib *et al.* (11) demonstrated increased tumor response to vincristine after a 24-h pretreatment with bryostatins 1 in SCID mice bearing the WSU-DLCL2 xenografts. Similar results have been shown in a xenograft model bearing a human Waldenstrom's macroglobulinemia tumor (12). The response was attributed in part to the down-regulation of P-glycoprotein and *mdr1* RNA expression induced by bryostatins 1 in the lymphoma cells.

On the basis of the above preclinical data, we embarked on a Phase I trial of the combination of bryostatins 1 and vincristine mimicking the preclinical model of a 24-h exposure to bryostatins 1 followed by exposure to vincristine. Objectives of this study included determination of DLT, the MTD, and observation for any preliminary evidence of antitumor activity in patients with relapsed B-cell malignancies. Laboratory correlative studies were also performed looking for evidence of apoptosis by three methods (cell morphology after acridine orange, flow cytometry, and PARP cleavage). We also measured various cytokines (IL-2, IL-2 receptor, TNF- $\alpha$ , and IL-6) during the initial 36 h of treatment to examine the potential immunomodulatory effects of bryostatins 1. Preclinical studies and early clinical trials of single-agent bryostatins 1 had suggested modulation of these cytokines.

## MATERIALS AND METHODS

**Study Population.** Patients with biopsy proven B-cell malignancies, including CLL, NHL, and MM were eligible. Patients with CLL required failure of an alkylating agent-containing regimen as well as fludarabine-based therapy. Patients with NHL were enrolled only after failure of all possible therapy with curative intent. Patients with MM must have received at least one prior chemotherapy regimen and not be eligible for a dose intensification treatment approach. Prior vincristine therapy was allowed. Prior stem cell or BM transplantation was permitted. Patients with HIV-related lymphoma were ineligible. Other eligibility criteria included an Eastern

Cooperative Oncology Group performance status of 0, 1, or 2 and a life expectancy of >12 weeks. Eligibility organ function criteria included: hematological function (WBC  $\geq 3000/\mu\text{l}$ , absolute neutrophil count  $\geq 1500/\mu\text{l}$ , platelets  $\geq 50,000/\mu\text{l}$ , hemoglobin > 8.5 g/dl); hepatic function (bilirubin  $\leq 1.5$  mg/dl, aspartate aminotransferase/alanine aminotransferase  $\leq 2\times$  upper limit of normal); and renal function (creatinine  $\leq 2.0$  mg/dl and/or actual creatinine clearance  $\geq 40$  ml/min/1.73 m<sup>2</sup>). Normal coagulation parameters were not required, however, patients were not to have any clinical evidence of a bleeding diathesis. Patients were followed every 2 weeks for the first 6 months on therapy and then every 3 weeks thereafter. Restaging was performed every two cycles.

**Treatment Schedule.** Bryostatins 1 was administered through a central venous catheter as a single 24-h i.v. infusion on day 1 of treatment. At the EOI, bolus vincristine was given. Treatments were repeated every 2 weeks for a minimum of one cycle (1 month) or until disease progression. Unacceptable toxicity after any number of cycles would terminate treatment for that patient. Patients who had completed six cycles of treatment (6 months) were then treated at 3-week intervals. In this situation, a cycle was considered to be 6 weeks rather than 4 weeks.

Dose escalation is depicted in Table 1. Cohorts of 3–6 patients were treated at each dose level. Vincristine was increased at dose level 2 to 1.4 mg/m<sup>2</sup> (maximum total dose, 2 mg) and then kept constant. In case of vincristine-associated neuropathy (grade 3 or higher), one dose reduction to 0.5 mg/m<sup>2</sup> was allowed; if symptoms precluded additional use of vincristine, patients with stable or responding disease were allowed to continue on bryostatins 1 alone. DLT was defined as any grade 3 nonhematological or grade 4 hematological toxicity. If DLT was observed in 2 of 3–6 patients enrolled at a specific dose level during cycle 1, that dose-level was considered the DLT level. A total of 6 patients was treated at the dose below the DLT level, thus establishing the MTD.

**Formulation.** Bryostatins 1 was supplied by the Division of Cancer Treatment, Diagnosis and Centers, NCI. This was supplied as a two-part formulation. The kit included a 6-ml flint vial containing 0.1 mg of bryostatins 1 as a white lyophilized cake or powder and 5 mg of povidone USP lyophilized from 40% *t*-butanol and a 2-ml flint vial containing 1 ml of sterile PET. The lyophilized powder was reconstituted with 1 ml of the PET diluent. After swirling the vial completely to dissolve the

contents, the resulting solution was further diluted with 9 ml of 0.9% sodium chloride injection, USP. This results in a 10  $\mu\text{g/ml}$  solution. Polyvinylchloride bags were not used because plasticizer can be leached with consequent absorption. The bryostatatin solution was further diluted with 0.9% sodium chloride to a concentration of 0.15 to 0.75  $\mu\text{g/ml}$  in a glass or polyolefin container.

**Response and Toxicity Criteria.** Toxicities were graded according to the NCI Common Toxicity Criteria, version 1.0 (13). Specific attention was given to myalgia as single-agent studies of bryostatatin 1 reported this symptom as being dose limiting. For myalgias, the myalgia toxicity grading scale was used. In this scale, grade 1 myalgia is defined as mild pain not interfering with daily activities; grade 2 is moderate pain in which pain or the analgesics produce some interference with daily activities; grade 3 is defined as severe pain in which the pain or analgesics severely interfere with daily activities; grade 4 is defined as disabling pain. The standardized response to therapy as outlined by the NCI-sponsored Working Group on CLL was used (14); for MM, the criteria for response were those described by Blade *et al.* (15); and for NHL, standard Eastern Cooperative Oncology Group response criteria were used (16).

**Methods for Correlative Studies.** Peripheral blood and BM were obtained for correlative studies. If the pretreatment BM demonstrated tumor involvement, a second BM aspiration was performed 6 h after the vincristine injection of cycle 1 for apoptosis assays. In all patients, peripheral blood was obtained for cytokine and apoptosis assays at the following time points: pretreatment (T 0); 6 h (T +6 h); 24 h (end of Bryostatatin 1 infusion, T +24 h); and 6 h after bolus vincristine administration (T +30 h).

**Isolation of PBMC and BM Leukocytes.** PBMCs and BM mononuclear cells were separated from  $\sim 10$  ml of fresh blood samples from a single individual by centrifugation on Percoll-Paque (Pharmacia, Uppsala, Sweden) at 325 g for 25 min. Platelets were removed by overlay of the cell suspension on heat-inactivated fetal bovine serum followed by centrifugation at  $85 \times g$  for 5 min. PBMCs were washed twice in cold PBS, and dry cell pellets were stored at  $-80^\circ\text{C}$  for subsequent gels and Western blots.

**Acridine Orange Staining.** An aliquot of 10- $\mu\text{l}$  stock solution (100  $\mu\text{g/ml}$  acridine orange prepared in PBS) was added to 100  $\mu\text{l}$  of single-cell suspension. Immediately thereafter, a minimum of 100 cells were counted under a fluorescence microscope. Cells with condensed chromatin in apoptotic bodies and cell membrane blebbing were counted as acridine orange positive (indicative of apoptosis), whereas cells with homogenous chromatin were counted as acridine negative.

**PARP Cleavage Western Blotting.** Cells were lysed and sonicated in a solution comprised of 0.5% sodium deoxycholate, 0.2% SDS, 1% Triton X-100, 5 mM EDTA, 10  $\mu\text{g/ml}$  aprotinin, 10  $\mu\text{g/ml}$  leupeptin, and 1 mM phenylmethylsulfonyl fluoride (all reagents were from Sigma Chemical Co., St. Louis, MO). Samples containing 30–70  $\mu\text{g}$  of protein measured by Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA) were separated by SDS-PAGE consisting of a 5% (w/v) acrylamide stacking gel and a 12.5% (w/v) separating gel containing

0.1% SDS (5). The running buffer was comprised of 0.1% SDS, 25 mM Tris, and 250 mM glycine (pH 8.3). Electrophoretic fractionation was carried out at a constant current of 15 mA. Proteins were then electrotransferred onto an Immobilon p15 membrane (Milipore Corp., Bedford, MA). The filters were blocked with 5% BSA in PBS containing 0.1% Tween 20 (PBS-Tween) for 5 min and then incubated overnight at  $4^\circ\text{C}$  with primary antibody at a concentration of 1  $\mu\text{g/ml}$  in blocking solution. After washing in PBS-Tween, the filters were incubated overnight at  $4^\circ\text{C}$  in horseradish peroxidase-conjugated anti-immunoglobulin (1:1000). After three washes in PBS-Tween, bands were visualized with enhanced chemiluminescence reagent and subsequent exposure to hyperfilm-enhanced chemiluminescence (Amersham Life Science, Inc., Arlington Heights, IL). The monoclonal antibody to purified human PARP is an IgG1 $\kappa$  that shows specificity for the NAD-binding domain of PARP (PharMingen, San Diego, CA). Appearance on the Western blot of a  $M_r$  90,000 protein was indicative of PARP cleavage (18).

**Flow Cytometry.** Peripheral blood and/or BM mononuclear cells were isolated by our Stem Cell core facility as indicated above. For lymphoma cases, cells were stained with extracellular domain-conjugated anti-CD19 and RD1-conjugated anti-CD5, and in the case of myeloma, cells were stained with antibodies against CD38 (phycoerythrin conjugated) and CD45 (adenomatous polyposis coli conjugated). All antibodies were purchased from Immunotech (Marseille, France). ECD is a phycoerythrin analogue, and RD1 is a tandem conjugate of Texas Red and ECD (Immunotech). These dyes fluoresce orange and red, respectively, upon excitation with 488 nm light. FITC-conjugated annexin V (Immunotech) was added according to manufacturers instructions 30 min before flow cytometry. Cell measurements were acquired with either a Beckman/Coulter (Miami, FL) Xcel or Elite cytometer using 488 nm of excitation and 530/25 nm, 575/30 nm, and 610 nm lp emission windows. Data were acquired as uncompensated fluorescence in list mode. The flow cytometric determination of apoptotic cells (annexin positive) was set empirically to the right of the major log-normally distributed peak of low annexin V-related fluorescence. This setting was extremely robust in that the gates could be set for the apoptotic fraction once and rarely moved and then only by a small fraction of channels. The intensity of marker staining was not as robust, which could be attributable to biology, cell preparation, or staining. All runs were controlled with fluorescence standard microspheres (Immunobrites; Beckman/Coulter), so the instrument performance was constant and known. The apoptotic fraction in normal lymphocytes was  $8 \pm 6\%$  (4 samples performed at widely spaced intervals). Winlist (Verity Software House, Topsham, ME) was used to analyze list mode data. In the text, cells expressing CD5 but not CD19 are referred to as CD5+; CD5-negative/CD19-positive cells as CD19+, and cells expressing both CD5 and CD19 as CD5/CD19++.

**Cytokines (IL-6, IL-2R, IL-2, and TNF- $\alpha$ ).** Human IL-2, IL-2R, IL-6, and TNF- $\alpha$  measurements on plasma were made using commercial kits (Quantikine HS; R&D Systems, Inc., Minneapolis, MN). These assays use the quantitative sandwich enzyme immunoassay technique. All samples were run in duplicate.

Table 2 Patient characteristics

	No. of Patients
Age (years)	59 (range 30–79)
Sex (M/F)	13/11
Total patients assessable	24
No. of courses/patient	
Median	4
Range	1–36
Histology	
CLL	2
MM	
With previous transplant	3
Without previous transplant	6
Low-grade lymphomas	
Follicular	3
Mantle cell	3
Small lymphocytic	1
Aggressive lymphomas	
Diffuse large cell lymphoma	6

## RESULTS

**Patients.** Twenty-five patients were enrolled onto this study from June 1998 to May 2001. Patient characteristics are listed in Table 2. Twenty-four patients were assessable for toxicity and response. One patient at DLT dose level was not assessable because although he did meet eligibility, the day-1 labs showed a low absolute neutrophil count, and the patient rapidly developed sepsis that was believed to be disease related. Six of the 13 NHL patients and 4 of the 9 MM patients had prior stem cell transplantation. Median number of prior chemotherapy regimens was 2 (range, 1–5). Twenty of the 24 patients had received prior vincristine. Twenty-four patients have received a total of 182 cycles of therapy.

**Toxicity.** Toxicities are depicted in Table 3. The myalgia was generally not cumulative except at the DLT level. All patients at the DLT level required dose reduction either because of the occurrence of DLT or worsening myalgia with ongoing therapy. In addition, 1 patient at the MTD required dose reduction of bryostatin 1 because of cumulative myalgia. Five patients required dose reduction in vincristine (to 0.5 mg/m<sup>2</sup>) because of cumulative sensory polyneuropathy (dose reductions during cycles 6, 7, 2, 7, and 5 in these 5 patients, respectively). The polyneuropathy was reversible and improved in all patients after dose reduction. There seemed to be no correlation between the need for dose reduction of the vincristine and the bryostatin 1 dose. No other drug-related toxicities were observed. Of importance was the relative lack of any significant myelotoxicity in this heavily pretreated population despite the eligibility criteria that allowed for low baseline hematological counts. This allowed for treatment of patients with relapses after stem cell transplantation.

**Response.** Responses and prolonged stable disease (>6 months) were seen in 10 patients (Table 4). Two patients had a confirmed complete response. One patient had primary refractory DLCL and had failed induction CHOP therapy (persistent marrow involvement by DLCL after six cycles of CHOP). He refused a dose intensification strategy and was enrolled on this study. Repeat BM after eight cycles demonstrated clearing of his BM on microscopic examination. He relapsed 24 months later

and was salvaged with rituximab with which he also had a complete response. A patient with DLCL who relapsed within several months of high-dose therapy and peripheral blood stem cell transplantation and had failed rituximab obtained a complete response based on the computed tomography and PET scan results. He remains in remission > 3 years later and is now off therapy for 18 months with no evidence recurrence. Another patient with relapsed mantle cell lymphoma and had progressed on prior therapy obtained a partial response and remained in remission for 20 months. A patient with low-grade follicular lymphoma obtained a partial response for 18 months and then progressed with transformation into a DLCL. Another patient with small lymphocytic lymphoma is continuing in partial remission for >2 years. In all 5 patients that experienced a response, tumor shrinkage was progressive with a decline in measurements seen after each restaging procedure. Three prolonged stable disease (>6 months) were seen in MM patients. Two additional patients with NHL also had prolonged stable disease (1 with low-grade lymphoma and 1 with a diffuse large-cell histology). Time to tumor response was very late compared with standard cytotoxic chemotherapy. In the 5 patients with either a complete or partial response the time to response was 4, 6, 8, 8, and 13 months, respectively.

**Cytokines.** There was a significant increase in IL-6 during the bryostatin infusion (Table 5). This increased additionally with the vincristine injection. EOI IL-6 levels were statistically higher than preinfusion levels ( $P = 0.01$ ). Six-h postvincristine levels were higher than prebryostatin 1 infusion ( $P = 0.001$ ) and higher than EOI bryostatin 1 IL-6 levels ( $P = 0.001$ , Fig. 1). This increase was across all dose levels and was not related to bryostatin 1 dose. No changes were seen in the other cytokines. For IL-2, 20 of the 96 measured time points were below the limit of detection of the assay, and therefore, no statistical analysis was performed.

**Apoptosis Studies.** To assess the effect of treatment on programmed cell death as a marker of response, we evaluated apoptotic cell morphology using acridine orange staining, PARP cleavage by Western blotting, and annexin V binding by flow cytometry. Sequential analysis of the various time points showed no change in the number of cells staining positive for acridine orange. In addition, no significant cleavage of PARP was observed at the time points analyzed.

Fig. 2 shows the annexin V frequency dynamics of CD5+ cells in lymphoma patients. The majority of patients demonstrated a decrease in apoptotic frequency at the 6-h time point with a subsequent return to near baseline at 24 h (black solid line). Four patients did not have the 6-h time point drop in apoptotic frequency; 2 of these patients had a complete and partial response and 2 had prolonged stable disease (gray dashed line). Furthermore, 5 patients had an overall increase in apoptotic frequency by the 30-h time point. Four of these were the same patients that lacked the drop at 6-h time point. One additional patient who showed the 6-h drop then went on to show an overall increase in apoptotic rate. This patient also obtained a partial response. *t* tests were performed on the normalized data for T 0 versus 6 and 24 versus 6 h. The differences at 0 and 24 h were significant for the CD5+ population (0 versus 6 h,  $P < 0.01$ ,  $n = 13$ ; 6 versus 24 h  $P < 0.0025$ ). The CD19+ population data were not significant ( $P < 0.2$ ); how-

Table 3 Toxicities/dose level

Dose level	Bryostatin dose ( $\mu\text{g}/\text{m}^2$ )	No. of patients	Toxicity (no. of patients)											
			Cycle 1				Cycle 1				All cycles			
			Myalgia grade				Neutropenia				Neurosensory			
			1	2	3	4	3	4	1	2	3	4		
1	12.5	3	2					1			1			
2	16.5	3	1					1		2			1	
3	22	3	1							1				
4	30	3	1							2	1			
5	40	3								2				
6	50	6	1	1				2		3	1			
7	62.5	4		1	1				1	1	1			

ever, the number of samples that had significant CD19+ cells was lower than that for CD5+ ( $n = 7$  for 0 versus 6 h ( $P < 0.2$ ) and  $n = 9$  for 6 versus 24 h ( $P < 0.1$ )). CD19+ cells were not very abundant in these samples (data were analyzed only for patients with  $>1\%$  CD19+ cells). In myeloma patients, no change in apoptotic frequency was detected in BM samples obtained at the indicated time points.

## DISCUSSION

This Phase I trial determined the MTD of bryostatin 1 and vincristine to be  $50 \mu\text{g}/\text{m}^2$  over 24 h and  $1.4 \text{ mg}/\text{m}^2$  (maximum total dose = 2 mg), respectively. The major side effect and DLT has been myalgia. This is consistent with other studies of bryostatin 1 (19–21). The use of vincristine does not seem to potentiate this effect. Furthermore, bryostatin 1 does not seem to increase the peripheral neuropathy associated with vincristine. The hematological tolerance of this combination makes it an attractive regimen to further study in patients with advanced and various B-cell malignancies, many of which have compromised marrow function because of extensive prior treatments. The majority of current regimens in development result in myelosuppression and increased risk of neutropenic fever, none of which were seen with the MTD of this regimen. This lack of myelosuppression was predicted by preclinical studies. In fact,

studies of bryostatin 1 *in vitro* shows that bryostatin 1 activates human neutrophils by binding to the phorbol receptor (22). Furthermore, inactive thymocytes from W/W<sup>v</sup> mice in coculture with W/W<sup>v</sup> BM shows stimulation of erythropoiesis in the presence of bryostatin. It has been suggested that bryostatin may be a useful clinical agent to stimulate hematopoiesis *in vivo* (23).

Of importance has been the preliminary evidence of anti-tumor activity seen in this relapsed patient population. Although the primary goal of Phase I studies is not to determine antitumor efficacy, it is encouraging to see several complete and partial responses, as well as prolonged stable disease during this study. The contribution of bryostatin 1 to these responses within this combination regimen cannot be determined in this Phase I study. However, the addition of bryostatin 1 to the CHOP regimen has also been shown to improve response in a CHOP-resistant human diffuse large-cell lymphoma xenograft model (24). Also interesting has been the fact that time to maximum response has been late (median of 8 months; range, 4–13 months) and not as traditionally seen with cytotoxic chemotherapy. This pattern of delayed response may favor an immunological mechanism of antitumor activity. This may have implications in terms of future evaluation of this regimen in which patients with stable disease should be given the opportunity to continue treatment to evaluate for response. Early withdrawal of patients from such a

Table 4 Antitumor activity: ( $n = 25$ ) (7/1/03)

	Tumor type	Dose level at which response occurred (bryostatin $\mu\text{g}/\text{m}^2/24 \text{ h}$ )	Time to disease progression from cycle 1	Prior therapies before enrollment <sup>a</sup>
Complete response	DLCL	16.5	24 months	CHOP (primary refractory)
	DLCL	40	42+ months-continuing	CHOP, DHAP, Rituxin, HDC + PBSCT
Partial response	NHL (small lymphocytic)	62.5	27+ months-continuing	CHOP
	NHL (follicular cell)	50	15 months	CVP, rituxin, fludarabine
	Mantle cell lymphoma	50	20 months	CHOP, Rituxin, DHAP
Stable disease ( $\geq 6$ months)	MM	12.5	6 months	VAD, MP, VBMCP, high dose cyclophosphamide
	NHL (diffuse large cell)	12.5	6 months	CHOP, DHAP, HD + PBSCT
	MM	16.5	6 months	MP
	NHL (diffuse large cell)	30	12 months	CHOP, DHAP
	MM	30	48+ months-continuing	VAD, HDC + PBSCT

<sup>a</sup> DHAP, decadron, cytarabine, cisplatin; MP, melphalan, prednisone; HD + PBSCT, high-dose chemotherapy and peripheral blood stem cell transplantation; VAD, vincristine, doxorubicin, dexamethasone; VBMCP, vincristine, BCNU, cyclophosphamide, melphalan, prednisone; CVP, cyclophosphamide-vincristine-prednisone.

Table 5 Analysis of IL-6, TNF- $\alpha$ , IL-2, IL-2R before, during, and after bryostatatin-1 and vincristine administration (mean  $\pm$  SD)

	Time points analyzed			
	Pre-bryostatatin infusion	6 h into bryostatatin infusion	End of bryostatatin infusion (before vincristine)	6 h post vincristine injection
IL-6	7.7 $\pm$ 17.4	5.1 $\pm$ 2.5	25.4 $\pm$ 78.4	33 $\pm$ 113.3
TNF- $\alpha$	7.7 $\pm$ 8.6	6.8 $\pm$ 7.8	10.8 $\pm$ 16.6	8.5 $\pm$ 8.2
IL-2R	4473 $\pm$ 5759	4196 $\pm$ 5804	4647 $\pm$ 6096	4531 $\pm$ 5747

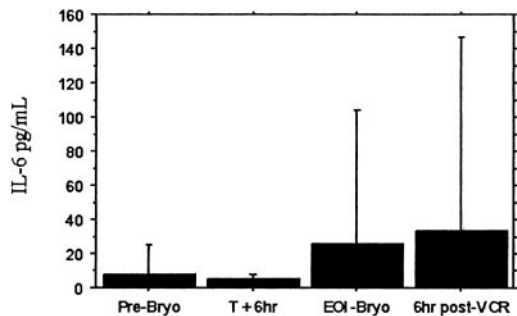


Fig. 1 Serum IL-6 at various time points during and after bryostatatin 1 infusion. Pre-bryostatatin (Bryo) versus T + 6 h,  $P = 0.01$ . Pre-bryo versus EOI-bryo  $P = 0.001$ . Pre-bryo versus 6-h postvincristine (VCR),  $P < 0.0001$ . EOI-Bryo versus 6-h post-VCR,  $P = 0.008$ .

regimen because of lack of response may underestimate the efficacy of this regimen. Although the majority of Phase I and II studies of bryostatatin 1, as a single agent, have failed to demonstrate any antitumor effect, the value of such a class of agent may reside in its effect on other chemotherapeutic agents as indicated by preclinical studies. As the majority of patients on this trial were previously treated with vincristine-containing regimens and had subsequently progressed, the activity seen here may suggest a reversal of vincristine resistance. Indeed, it has been suggested that bryostatatin down-regulates mdr protein, and this may be one mechanism by which it can reverse vincristine resistance (11).

The mechanism and implications for increase in serum IL-6 is unclear. *In vitro* studies show that bryostatatin both alone and in conjunction with IL-1 $\alpha$  induces secretion of granulocyte colony-stimulating factor and other cytokines, including IL-6 from marrow stromal cells (25). Furthermore, bryostatatin 1 potently activates human monocytes to produce cytokines, including IL-6 (26). One theoretical concern would be its effect on myeloma cell proliferation given its known implication in this disease. However, 1 patient with myeloma had prolonged stable disease after having relapsed after BM transplantation, suggesting that it may have no clinical implication, although this would need to be additionally explored in clinical trials and in preclinical models. At least one other clinical study has confirmed an increase in serum IL-6 during the early phases of administration of bryostatatin 1 (27). In this study, increases in IL-6 were reported at 2 and 24 h of starting a bryostatatin infusion (27).

Other studies also demonstrated an increase TNF- $\alpha$ , IL-2, and IL-2R with single-agent bryostatatin 1, something we were unable to demonstrate in our trial (9, 27).

One of the most interesting properties of the bryostatins is the modulation of apoptosis through possibly multiple pathways, including phosphorylation of bcl-2 (28). The apoptotic assays failed to demonstrate apoptosis by either acridine orange staining or PARP cleavage. This may be a true finding or a technical problem related to the assays such as the type of assay used or the timing of sample collection. The sample collection timing was based on preclinical models. There was a statistically significant depression in the apoptotic frequency of CD5+ cells at the 6-h time point after therapy that in the average case rebounded in samples taken 18 h later. This may be consistent with the known effect on the induction of PKC by bryostatatin 1 (seen within the first few hours of exposure; Ref. 6) followed by down-regulation after more prolonged exposure (*i.e.*, 24 h; Ref. 7). In the 5 patients with an increase in apoptotic frequency in CD5+ cells by annexin V staining, a clinical response or prolonged stable disease was seen. It is important to emphasize that the apoptotic studies are exploratory in nature and need confirmation in additional studies of this regimen.

In conclusion, we have determined the recommended Phase II dose of bryostatatin 1 as a 24-h infusion followed by bolus vincristine, repeated every 14 days. Myelotoxicity is minimal and myalgia is dose related. There has been evidence of antitumor activity during this trial. Serum IL-6 is increased with this regimen. Future studies of this combination in various B-cell malignancies are warranted.

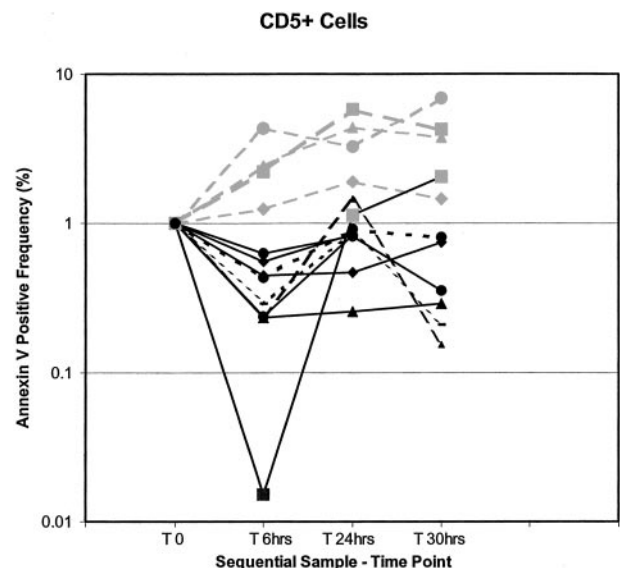


Fig. 2 Annexin V frequency dynamics in peripheral blood CD 5+ cells in lymphoma patients. For 4 patients, the apoptotic frequency (apf) increased continuously (gray dashed lines); 5 patients showed sustained apf above T = 0. For the remainder (—), the direction was continuously at or below the initial value. There is a marked decrease in apoptotic frequency in the majority of samples at the 6-h sample with return to baseline at 24 h.

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Afshin Dowlati, Hillard M. Lazarus, Paul Hartman, et al.

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