Phase I Trial and Correlative Laboratory Studies of Bryostatin 1 (NSC 339555) and High-Dose 1-B-D-Arabinofuranosylcytosine in Patients with Refractory Acute Leukemia

Louise H. Cragg, Michael Andreeff, Eric Feldman, John Roberts, Anthony Murgo, Mary Winning, Mary Beth Tombes, Gail Roboz, Lora Kramer, and Steven Grant

Division of Hematology/Oncology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23284 [L. H. C., J. R., M. W., M. B. T., L. K., S. G.]; Weill Medical College, Cornell University, Ithaca, New York 14853 [E. F., G. R.]; M. D. Anderson Cancer Center, University of Texas Southwestern Medical Center, Houston, Texas 75390-8593 [M. A.]; and the Cancer Therapy and Evaluation Program, National Cancer Institute, Bethesda, Maryland 20892 [A. M.]

ABSTRACT

A Phase I trial has been conducted in patients with refractory/refractory acute leukemia in which escalating doses of the protein kinase C (PKC) activator and down-regulator bryostatin 1 (NSC399555), administered as a 24-h continuous infusion on days 1 and 11, were given immediately before and after a split course of high-dose 1-β-D-arabinofuranosylcytosine (HiDAC; 1.5 g/m^2 every 12 h) administered on days 2 and 3, and 9 and 10. The bryostatin 1 maximally tolerated dose (MTD) was identified as 50 μg/m^2, with myalgias representing the major dose-limiting toxicity (DLT). Other DLTs included prolonged neutropenia and thrombocytopenia, and hepatotoxicity. Of the 23 patients who completed their course of therapy and were fully evaluable for response, the large majority of whom had unfavorable prognostic characteristics, 4 complete remissions (CRs) were obtained. An additional 3 patients were treated at a 3 g/m^2 ara-C (1-β-D-arabinofuranosylcytosine) dose level to determine whether this HiDAC dose could be administered in conjunction with bryostatin 1. All 3 of these patients experienced DLT, and this dose was considered above the MTD. However, one of the latter patients, who was heavily pretreated, also achieved a CR that persisted 5+ months without maintenance. Finally, 1 patient post-HiDAC and autologous bone marrow transplantation achieved a 5+ month leukemia-free survival although she did not meet the criteria for a CR because of persistent transfusion requirements. Correlative laboratory studies performed on blasts from 9 patients revealed that in vivo administration of bryostatin 1 resulted in variable effects on total blast PKC activity, including decreases in 4 samples, increases in 2, and no change in 3. Previous in vivo bryostatin 1 exposure also exerted disparate effects on the extent of apoptosis observed in blasts exposed to ara-C ex vivo, although increases were noted in a subset of patient samples. Interestingly, in vivo administration of bryostatin 1 by itself induced lethality in some patient specimens. No clear relationship between the in vivo effects of bryostatin 1 on blast PKC activity and the extent of ara-C-related apoptosis that occurred ex vivo was apparent. Together, these findings demonstrate that bryostatin 1 can be safely administered as a continuous infusion before and after a split course of HiDAC in patients with refractory leukemia, and identify the bryostatin 1 MTD as 50 μg/m^2 when given by this schedule. Furthermore, the achievement of several CRs in the setting of a Phase I trial in which many patients had particularly high-risk features (e.g., short initial remission, previous HiDAC or autologous bone marrow transplantation, and multiple previous salvage regimens) suggests that this regimen has activity in acute leukemia and warrants additional investigation.

INTRODUCTION

ara-C is a nucleoside analogue, which is among the most active agents available for the treatment of AML (1). It is transported across the cell membrane by a facilitated nucleoside diffusion mechanism and converted to its nucleotide form by the pyrimidine salvage pathway enzyme, deoxycytidine kinase (2). Ara-C is ultimately metabolized to its active derivative, ara-C 5′-triphosphate, which inhibits DNA polymerase α and is also incorporated into DNA strands, resulting in inhibition of chain initiation and elongation, and chain termination (3). Like most cytotoxic drugs, ara-C induces mitochondrial injury (e.g., cytochrome c release) and apoptosis, particularly in leukemic cells (4). When given according to conventional dose schedules (e.g., 4) the abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; PKC, protein kinase C; MTD, maximally tolerated dose; DLT, dose-limiting toxicity; 7-AAD, 7-amino actinomycin D; HiDAC, high-dose 1-β-D-arabinofuranosylcytosine; TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling; PBMC, peripheral blood mononuclear cell; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; CR, complete remission.

Received 1/15/02; revised 4/16/02; accepted 4/24/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by awards CA63753, CA92950, and CA77141 from the National Cancer Institute/NIH, and award 6630-1 from the Leukemia and Lymphoma Society of America. Portions of this work have been presented in preliminary form at the American Society of Hematology, San Francisco, CA, December 4–9th, 2000.

2 These authors contributed equally to this work.

3 To whom reprint requests should be sent at Hematology/Oncology, Medical College of Virginia, MCV Station Box 230, Richmond, VA 23298. Phone: (804) 828-5211; Fax: (804) 828-8079; E-mail: stgrant@hsc.vcu.edu.
100–200 mg/m²/day by continuous infusion × 5–7 days), plasma levels of 0.1–1.0 μM are obtained (5). However, it has been found that when given as a high-dose bolus infusion (e.g., 1–3 g/m² over 1–3 h every 12 h × 3–6 days), plasma ara-C levels in the 10–100 μM range are achievable (6). The observation that some leukemic patients who have failed conventional dose ara-C respond to high-dose regimens (7) suggests that at least some forms of ara-C resistance can be circumvented by increasing the concentration of ara-C to which leukemic cells are exposed in vivo.

Bryostatin 1 (NSC 339555) is a macrocyclic lactone PKC activator derived from the marine bryozoan Bugula nerina. It exerts antiproliferative effects against a variety of human tumor cells both in vitro as well as in vivo (8–10). Bryostatin 1 appears to be particularly active against malignant hematopoietic cells. For example, it is capable of inducing differentiation in human myeloid leukemia cells (11, 12), although it is less effective in this regard than the tumor-promoting phorboid phorbol 12-myristate 13-acetate (13, 14). Bryostatin 1 also inhibits the clonogenic growth and self-renewal capacity of human leukemic myeloblasts (15, 16). Several Phase I trials of bryostatin 1 have been conducted in humans using a variety of schedules, including a 24-h continuous infusion every week for 8 weeks and a bolus infusion every week × 3 on a monthly basis (17–19). The MTD for bryostatin 1 has been identified as 37.5–50 μg/m², depending on the schedule; the major DLT has been myalgias (18–20). Other toxicities have included fatigue, central nervous system disturbances, abnormal liver function tests, phlebitis, and fevers (17–20). When administered monthly as a 120-h continuous infusion, bryostatin 1 doses as high as 120 μg/m² have been found to be tolerable (21). Phase I activity has been observed in patients with non-Hodgkin’s lymphoma, melanoma, and ovarian carcinoma (19–21).

Preclinical studies have suggested that in addition to its intrinsic antitumor activity, bryostatin 1 alters the threshold for cytotoxic drug-induced apoptosis, a capacity that may stem from its effects on PKC (22). PKC is a ubiquitous Ca²⁺- and lipid-dependent serine threonine kinase, consisting of at least 13 isoforms, which is intimately involved in cell proliferation, differentiation, and stimulus-response coupling, among other activities (23). Whereas acute exposure of cells to bryostatin 1 activates PKC, chronic exposure down-regulates the enzyme (24), possibly by promoting PKC ubiquitinization and proteasomal degradation (25). The bulk of evidence suggests that PKC activation opposes apoptosis, particularly in hematopoietic cells (26), a notion supported by the observations that pharmacologic PKC inhibitors are potent inducers of apoptosis (27, 28), and may also lower the threshold for apoptosis triggered by cytotoxic drugs, including ara-C (29, 30). Previous studies from our laboratory have shown that combined exposure to bryostatin 1 and ara-C is highly inhibitory to the self-renewal capacity of primary human leukemic blasts (16), and that pretreatment of human promyelocytic leukemia cells (HL-60) with bryostatin 1 increases apoptosis induced by ara-C, a phenomenon that is temporally related to PKC down-regulation (22). Furthermore, bryostatin 1 has been shown to be capable of reversing, at least in part, resistance of these cells to ara-C-induced lethality conferred by overexpression of the antiapoptotic protein Bcl-2 (31). Interestingly, in human monocytic leukemic cells (U937), which, in contrast to HL-60 cells, are at least partially responsive to bryostatin 1-induced maturation, the sequence ara-C followed by bryostatin 1 was associated with maximal potentiation of apoptosis (32). The latter phenomenon may reflect disruption of leukemic cell maturation by prior exposure to a DNA-damaging agent, a phenomenon that has been described previously (33). In any case, such preclinical findings raise the possibility that bryostatin 1 and ara-C interact synergistically in a sequence-dependent manner to induce apoptosis in human leukemia cells through a process that may involve PKC down-regulation, dysregulation of leukemic cell maturation, or a combination of these events.

In a recently completed Phase Ib trial, we reported that administration of a fixed dose of bryostatin 1 (e.g., 25 μg/m²) according to three schedules i.e., a 1-h infusion, a 24-h infusion, and a split course (12.5 μg/m² as a 30-min infusion) on days 1 and 4, resulted in PKC down-regulation in a surrogate hematopoietic target tissue (e.g., normal PBMNCs) in a subset of patients (34). Such a finding raised the possibility that in vivo administration of bryostatin 1 might enhance the activity of HiDAC in leukemic blasts, analogous to preclinical results, if indeed PKC down-regulation promoted ara-C-mediated apoptosis in these cells (30). To test this possibility, a Phase I trial of bryostatin 1 administered in conjunction with HiDAC was designed for patients with refractory/refractory acute leukemia. Because a 24-h continuous infusion was at least as effective as the other schedules in down-regulating PBMC PKC activity (34) and closely mimicked exposures shown to enhance ara-C-related apoptosis in preclinical studies (22, 33), this schedule was selected for the present trial. The primary goal was to define the MTD of bryostatin 1 that could be administered before and after a course of HiDAC, and to define the dose-limiting toxicities of this regimen. Ancillary goals were to determine whether and to what extent in vivo administration of bryostatin 1 could down-regulate total PKC activity in primary leukemic blasts, and increase their ex vivo susceptibility to ara-C-induced apoptosis.

MATERIALS AND METHODS

Drug Formulation

Bryostatin 1 (NSC 339555) was supplied by the Cancer Therapy and Evaluation Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD. It was stored at 4°C in flint vials containing 0.1 mg of bryostatin and 5 mg of povidone USP lyophilized from 40% t-butanol. Material was reconstituted in 1 ml of sterile PET (60% polyethylene glycol 400, 30% ethanol, and 10% Tween 80) diluent. The resulting solution was diluted additionally with 9 ml of 0.9% sodium chloride. Tubing for the drug infusion was primed with bryostatin 1 in the PET formulation diluted with sodium chloride to minimize adsorption of drug. Bryostatin was administered by syringe pump via a central line with infusion of 2 liters of normal saline.

Ara-C (Cytosar; Upjohn, Kalamazoo, MI) was supplied in 100 and 500 mg vials, 1 and 2 g vials, and as a 20 mg/ml liquid preparation that requires no reconstitution. Ara-C is reconstituted with preservative-free sterile water for injection and diluted in 250 mg of 5% dextrose.
e.g., (22), whereas in cells at least partially responsive to bryostatin sequence bryostatin 1 followed by ara-C may maximize apoptosis.

For in vitro studies, ara-C hydrochloride was purchased from Sigma Chemicals (St. Louis, MO), formulated in sterile water at a concentration of $10^{-2} \text{ M}$, and stored frozen until use. Subsequent dilutions were made in RPMI 1640.

**Study Design and Rationale**

The study design was based on the regimen of Capizzi et al. (35), in which L-asparaginase was administered after each fourth infusion of HiDAC (3 g/m² every 12 h × 4 on days 1 and 2, and 9 and 10). In the present design, an initial HiDAC dose of 1.5 g/m² was selected based on evidence that such a dose may be better tolerated and as effective as higher doses (36). In addition, based on preclinical evidence that in cells unresponsive to bryostatin 1-induced maturation (e.g., HL-60), the sequence bryostatin 1 followed by ara-C may maximize apoptosis (22), whereas in cells at least partially responsive to bryostatin 1 maturation (e.g., U937), the sequence ara-C followed by bryostatin 1 may result in maximal cell death (32), the sequence of these agents varied at the beginning and end of the regimen. Thus, all of the patients initially received a 24-h infusion of bryostatin 1 (day 1) followed, 1 h after the end of the infusion, by 4 doses of HiDAC (days 2 and 3). On days 9 and 10, patients received the second course of HiDAC, this time followed by bryostatin 1 at the same dose administered on day 1. The central concept underlying this design was that leukemic blasts may respond heterogeneously to these agents and that differentiation-unresponsive blasts, like HL-60 cells, may be most sensitive to the sequence bryostatin 1→HiDAC, whereas maturation-competent blasts, like U937 cells, may respond best to the sequence HiDAC→bryostatin 1. Although this study design did not allow direct comparisons to be made between the effects of the two sequences on toxicity and antileukemic activity, it was felt that based on the theoretical benefits, this therapeutic strategy was justified. Finally, at the end of the study, 3 additional patients were treated with 50 µg/m² bryostatin 1 in conjunction with HiDAC (3 g/m² every 12 h × 4) to determine whether administration of these drug doses was feasible. However, all 3 of the patients experienced DLT, and these doses were considered above the MTD. A schematic diagram of the protocol design is shown in Fig. 1.

**Patient Eligibility**

Patients with primary refractory or relapsed acute leukemia (AML or ALL), or refractory or relapsed lymphoblastic lymphoma were eligible for this study. In addition, patients with chronic myelogenous leukemia in myeloid blast crisis who were either treated previously or untreated were considered eligible for protocol entry. Eligibility criteria also included Karnosky performance status ≥60; age ≥18 years (patients receiving 3 g/m² ara-C must have been ≥60 years of age); calculated or actual creatinine clearance ≥40 ml/min (for patients receiving 3 g/m² ara-C, the creatinine clearance must have been ≥60 ml/min); total bilirubin ≤2 mg/dl (excluding patients with Gilbert's syndrome); aspartate aminotransferase/alkaline phosphatase ≤2.5 × upper limit of normal; and patients must have recovered from the major toxic effects of previous therapy. Patients were excluded for the following: systemic chemotherapy within 3 weeks before beginning study treatment (except for hydroxyurea, which needed to be discontinued 24 h before therapy); clinically significant pulmonary disease, previous significant ara-C-related cerebellar toxicity; poor medical risks because of nonmalignant systemic disease; active, uncontrolled, serious infection; pregnancy; and medical status that would make it difficult to interpret cortical or cerebellar neurological toxicity. The protocol was approved by the institutional review boards of each of the participating institutions. Informed consent was obtained from all of the patients, who were enrolled without restriction related to gender or race.

**Drug Administration and Dose Escalation**

Bryostatin was administered as a 24-h continuous infusion, at a starting dose of 12.5 µg/m². After completion (1 h) of the bryostatin infusion, four doses of ara-C (1.5 g/m² i.v. over 3 h every 12 h) were given. After a 7-day interval, four additional doses of ara-C, also at 1.5 g/m² i.v., were given beginning on day 9. A second 24-h continuous infusion of bryostatin, given at the same dose as that given on day 1, was initiated 60 min after completion of the last ara-C dose. At least 3 patients were treated at each dose level to evaluate toxicity. If one or more patients experienced a DLT, the cohort was expanded to 6 patients. If 33% of patients then experienced DLT, the dose was presumed to be the MTD and would be recommended for Phase II trial. If >33% of patients experienced DLT, the dose was presumed to exceed the MTD and the subsequent cohort was treated at a lower intermediate dose. In the absence of any DLT, the bryostatin dose was increased in increments of 12.5 µg/m² (e.g., to levels of 25.0, 37.5, and 50.0 µg/m²). Once the MTD of bryostatin given in combination with 1.5 g/m² ara-C was determined, the ara-C dose was escalated to 3.0 g/m² in a cohort of patients.

**Continuation Therapy**

Patients who achieved a complete response were eligible to receive up to 6 monthly courses of an attenuated schedule of bryostatin and HiDAC in which the bryostatin 1 dose was given at its initial dose before and immediately after HiDAC (1.5 g/m² ara-C every 12 h × 2) on days 1 and 9.
Assessment of Toxicity

Adverse events rated as possibly, probably, or definitely related to treatment were scored as toxicities according to the Cancer and Leukemia Group B Expanded Common Toxicity Criteria. Myalgia was evaluated as mild, (e.g., responsive to non-narcotic analgesics), moderate (e.g., requiring narcotic analgesics), or severe (unresponsive to narcotic analgesics). Any grade 3 toxicity was dose limiting except for infection, nausea, diarrhea, stomatitis, esophagitis/dysphagia, anorexia, bilirubin, transaminase, alopecia, dyspnea, hypotension, altered mood, insomnia, flu-like symptoms-malaise/fatigue, weight loss/weight gain, metabolic abnormalities, endocrine abnormalities, and myalgias. Persistence of grade 3 transaminase toxicity or hyperbilirubinemia >5× upper limit of normal for >7 days were considered DLTs. DLT was defined as any grade 4 toxicity, with the exception of hyperbilirubinemia, infection, hematologic toxicity, or end-organ toxicity clearly related to the hematologic toxicity. Additionally, persistence of grade 4 neutropenia or thrombocytopenia for >4 weeks was considered dose limiting.

Supportive Care

Patients received appropriate supportive care including the administration of antiemetics, alkalization of urine, and diagnostic evaluation and treatment of all of the documented or suspected infection. All of the patients received allopurinol (300 mg/day) during and for 1 week after treatment. Corticosteroid eye drops were administered during therapy to prevent ara-C-associated conjunctivitis. Menstruating women received hormone therapy to prevent ovulation and menstruation. Patients were transfused to maintain hemoglobin levels >8 g/dl and platelets >10 × 10^9/μl, or as clinically indicated. Hematopoietic growth factors were not permitted, with exceptions granted at the discretion of the principal investigator.

Patient Assessment

While in the hospital, patients were weighed and examined daily. Cerebellar toxicity was assessed before each ara-C dose. For grade 2 cerebellar toxicity, scheduled ara-C doses were to be omitted until recovery to ≤grade 1 toxicity. For grades ≥3 cerebellar toxicity, ara-C was to be discontinued and the second infusion of bryostatin canceled. Biochemistry, and renal and liver function tests were performed daily until day 12 and then every other day until discharge from hospital. Blood counts (complete blood count, differential and platelets) were obtained daily, and prothrombin time and activated prothrombin time, weekly, until absolute neutrophil count >1.5 × 10^9/μl and platelets >100 × 10^9/μl. A diagnostic bone marrow aspirate and biopsy was performed 6–8 days after the second bryostatin infusion ended or when peripheral WBC exceeded 1.5 × 10^9/μl, whichever was first. All of the patients were monitored for toxicity through at least day 24, or until the post-treatment bone marrow evaluation was done, whichever was latest. Patients were followed every 6 months until death for long-term survival assessment.

Response Assessment

Response assessment was based on criteria established by the National Cancer Institute workshop (37). CR was defined as follows: absolute neutrophil count ≥1.5 × 10^9/μl, platelets ≥100 × 10^9/μl, absence of leukemic blasts in peripheral blood, >20% cellularity of bone marrow with maturation of all of the cell lines, <5% leukemic blasts in the bone marrow, and absence of Auer rods. A partial remission was similarly defined except that the percentage of blast in the marrow was between 5 and 25%, and Auer rods could be present as long as the blast percentage was ≤5%.

Correlative Laboratory Studies

PKC Activity. Cells were obtained from the peripheral blood of patients before and immediately after the completion of the 24-h bryostatin 1 infusion. To avoid problems in interpretation because of sample heterogeneity, only specimens that contained ≥70% blasts were studied. Cells were diluted 1:4 in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 100 IU/ml of preservative-free heparin and layered over 10 ml of Ficoll-Hypaque (sp. grav 1.077; Sigma) in sterile 50-ml centrifuge tubes. The samples were centrifuged at 400 × g for 30 min at room temperature, after which the interface layers, containing primarily leukemic blasts, were extracted with a sterile Pasteur pipette. The blasts were either analyzed immediately for PKC activity or cell pellets placed in sterile 50-ml centrifuge tubes which were then directly transferred to a −80°C freezer. In separate studies, we have found the PKC activity in cells that are stored frozen for periods of up to 2 weeks remains within 15% of values obtained in nonfrozen specimens.

Total PKC activity in leukemic blasts was determined using a commercially available assay kit (Life Technologies, Inc.), which measures the extent of incorporation of 32P into myelin basic protein, as described previously in detail (34). Each assay was performed in triplicate, and comparisons were made between samples obtained before and after bryostatin 1 administration to assess effects on leukemic blast PKC activity.

Ex Vivo Apoptotic Responses to Ara-C. Specimens were obtained as above, except that in some cases, samples were shipped overnight at ambient temperature. We have found that handling in this way does not compromise viability in the large majority of samples. In all of the cases, only specimens that exhibited ≥90% viability by trypan blue exclusion were used in these studies. After isolating the mononuclear cell fraction, cells were suspended in RPMI 1640 containing 10% FCS at a concentration of 2 × 10^6 cells/ml, and transferred to sterile 25 cm^2 plastic tissue culture flasks (Corning, Corning, NY). To each flask was added the appropriate concentration of ara-C, after which the flasks were placed in a 37°C, 5% CO₂, and fully humidified incubator for the designated interval (i.e., 4 h). At the end of the incubation period, assessment of cell death was carried out as described below.

Morphological Assessment of Apoptosis. After treatment, aliquots containing blasts were subjected to cytocentrifu- gation using a Shandon cytocentrifuge and the slides stained with Wright-Giemsa as we have reported previously (22). Slides were viewed under light microscopy, and the percentage of cells exhibiting evidence of cell death (i.e., cell shrinkage, nuclear condensation, loss of nuclear architecture, and so forth) determined by scoring ≥12 randomly selected fields encompassing >500 cells.
**7-AAD Uptake and TUNEL Assay.** In some of the blast samples (four of seven), induction of cell death was confirmed by monitoring uptake of 7-AAD by flow cytometry as outlined previously in detail (38) or by examining TUNEL-stained cytospin preparations. The results of these studies were consistent (i.e., within 10%) of those obtained by morphological assessment of cell death in cytospin preparations.

**Statistical Analysis.** The significance of differences between experimental conditions was determined using the Student t test for unpaired observations.

**RESULTS**

**Patients**

Characteristics for the 30 patients enrolled in the study are depicted in Table 1. The median age was 60 years, and the majority of patients had a Karnofsky performance status of 80–100. The most frequent diagnosis was AML. Most patients were heavily pretreated, and many presented with resistant disease. Of note, 1 patient in first relapse and 1 patient in second relapse had failed an autologous bone marrow transplantation.

**Toxicity**

DLTs are described in Table 2. In general, toxicities seen were those expected during induction chemotherapy for acute leukemia. One patient at level one (bryostatin 12.5 μg/m²) had prolonged neutropenia after treatment that persisted until she relapsed 5 months later. It is noteworthy that this patient had previously undergone ABMT. At level 4 (bryostatin 50 μg/m²), 2 patients experienced grade 4 myalgias, defined as myalgias associated with severe pain despite narcotic analgesia. These began on days 5 and 6 of therapy, respectively. An MTD for bryostatin was thus identified. Two patients experienced reversible hepatic toxicity with grade 4 transaminitis in 1 patient and grade 4 hyperbilirubinemia in the other. A third patient developed grade 3 cardiac toxicity with a decrease in left ventricular ejection fraction requiring therapy. Of note, this patient had not had a baseline cardiac evaluation before study enrollment and had been treated previously with high-dose mitoxantrone. Nonhematologic grade 3 and 4 toxicities that were judged not to be DLTs included hyperbilirubinemia and various metabolic abnormalities, all of which were reversible (Table 3).

**Responses**

Hematologic responses to the HiDAC/bryostatin 1 regimen are summarized in Table 4. Seven patients were considered inequivalent for response to the regimen for the following rea-
Objective responses for patients treated at each of the ara-C/bryostatin 1 dose levels. Figures refer to the number of patients able to complete their course of therapy. Figures in parentheses correspond to the number of patients who, for various reasons, did not complete their course of therapy.

<table>
<thead>
<tr>
<th>Level</th>
<th>No. patients completed therapy</th>
<th>CR</th>
<th>Partial remission</th>
</tr>
</thead>
<tbody>
<tr>
<td>bryo 12.5 $\mu$g/m$^2$ and ara-C 1.5 g/m$^2$</td>
<td>6 (1)</td>
<td>1$^a$</td>
<td>0</td>
</tr>
<tr>
<td>bryo 25.0 $\mu$g/m$^2$ and ara-C 1.5 g/m$^2$</td>
<td>6</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>bryo 37.5 $\mu$g/m$^2$ and ara-C 1.5 g/m$^2$</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>bryo 50.0 $\mu$g/m$^2$ and ara-C 1.5 g/m$^2$</td>
<td>6 (1)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>bryo 50.0 $\mu$g/m$^2$ and ara-C 3.0 g/m$^2$</td>
<td>3 (3)</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$An additional patient treated at level 1 experienced a 5+ month leukemia-free interval but was not considered to have achieved an objective CR because of persistent transfusion requirements.

Table 4

Fig. 2 Effect of in vivo administration of bryostatin 1 on leukemic blast total PKC activity. Leukemic blasts were obtained from the peripheral blood of 10 patients before and immediately after completion of the initial bryostatin 1 infusion, administered at the designated dose. Blasts were lysed and total PKC activity determined as described in “Materials and Methods.” Values, expressed as the ratio of activity (pmol [3H]myelin basic protein/μg protein) obtained before and after administration of bryostatin 1, represent the means for triplicate determinations; bars, ± SD. *, significantly different from prebryostatin 1 values; $P \leq 0.05$.

Effects of in Vivo Administration of Bryostatin 1 on the ex Vivo Sensitivity of Leukemic Blasts to Ara-C. In view of evidence that in vitro exposure of leukemic cells to bryostatin 1 has been shown to enhance apoptotic responses to ara-C (22), an attempt was made to determine whether a similar phenomenon occurred in blasts exposed to bryostatin 1 in vivo. To address this issue, blasts were obtained before and immediately after the bryostatin 1 infusion, washed, exposed to 1 or 10 μM ara-C for 4 h, after which apoptosis was assessed by monitoring morphological correlates of cell death, including cell shrinkage, condensation of chromatin, nuclear dissolution, and loss of normal cytoarchitectural features, among others. In general, these results correlated well with other measures of apoptosis i.e., 7-AAD uptake (see below). Specimens from 7 patients were available for such analysis (Fig. 3). Several findings emerged from these studies. First, a 4-h exposure to 1 or 10 μM ara-C, in contrast to results obtained with leukemic cell lines (22, 34), was only marginally toxic to most specimens, possibly reflecting the relatively low S phase fraction of the majority of primary
AML blasts and/or the emergence of resistant cells. Second, in 3 samples (patients 3, 9, and 10), in vivo exposure to bryostatin 1 by itself resulted in a significant increase in cell death, whereas in one patient sample (patient 7), a slight reduction in apoptosis was noted. Effects of previous in vivo exposure of blasts to bryostatin 1 on ara-C sensitivity were heterogeneous, with a decrease in 2 specimens (patients 5 and 7), no change in 1 (patient 8), and increases for at least 1 ara-C concentration in 4 specimens (patients 3, 6, 9, and 10). In 3 of the latter 4 specimens, the increase in cell death could be attributed to the effects of bryostatin 1 alone. Two patient samples, which displayed a decrease in PKC activity post-bryostatin 1, exhibited an increase in ara-C-related apoptosis (patients 6 and 9), whereas 1 post-bryostatin 1 sample, which exhibited an increase in PKC activity, showed a reduction in apoptosis after ara-C exposure (patient 7). However, the small sample size precluded drawing conclusions regarding the relationship between changes in PKC activity and susceptibility to ara-C-associated apoptosis. In addition, for the reasons stated above, correlations between induction of apoptosis in blasts by bryostatin 1/ara-C and clinical outcome could not be

Fig. 3  Effect of in vivo administration of bryostatin 1 on ex vivo response of blasts to ara-C. Blasts were obtained from the peripheral blood of 9 patients immediately before (○) and after (●) administration of bryostatin 1. They were then incubated with either 1 or 10 μM ara-C for 4 h, after which cytocentrifuge preparations were obtained and stained with Wright-Giemsa. The slides were viewed under light microscopy, and the percentage of cells exhibiting apoptotic features determined by scoring at least 10 randomly selected fields encompassing ≥500 cells.
made. Nevertheless, these findings suggest that in vivo administration of bryostatin 1 induces lethality in at least some primary AML blasts in the presence or absence of ara-C.

Analysis of 7-AAD uptake by blasts obtained from a representative patient (patient 3) before and after bryostatin 1 administration is shown in Fig. 4, A and B. Increased uptake of 7-AAD has been found to correlate closely with apoptosis, particularly in malignant hematopoietic cells (38). The percentage of blasts displaying high uptake of 7-AAD was 10% pretreatment versus 29% in cells obtained immediately after the end of the bryostatin 1 infusion. The figures in parentheses represent values obtained after morphological assessment of cell death and are concordant with the results of 7-AAD analysis. Results from 3 additional patients revealed a close concordance between values obtained by morphological and flow cytometric analysis (39). Examination of TUNEL-stained cytospin preparations confirmed the increase in blasts exhibiting evidence of DNA strand breaks after bryostatin 1 treatment (Fig. 4C). These findings confirm the observation that in vivo administration of bryostatin 1 by itself can exert lethal effects toward primary human leukemic blasts in a subset of patients.

DISCUSSION

Preclinical evidence indicates a synergistic antileukemic interaction between bryostatin and ara-C, and suggests that this interaction stems, at least in part, from the ability of bryostatin to potentiate ara-C apoptosis (22, 34). Whereas ara-C is arguably the most effective single agent in AML (1), little information is available concerning the activity of bryostatin 1 in acute leukemia. Nevertheless, bryostatin 1 has shown in vitro cytotoxic activity toward both continuously cultured human myeloid leukemia cell lines (15), as well as against primary blast specimens (16). Moreover, the combination of ara-C and bryostatin 1 potently inhibits the self-renewal capacity of primary leukemic blasts (16), a response determinant that has been shown to correlate closely with clinical outcome (40). Although it is tempting to attribute this phenomenon to potentiation of apoptosis, it is important to note that both ara-C (41) and bryostatin 1 (11, 12) have been shown to induce leukemic cell maturation in vitro. Consequently, the loss of self-renewal capacity of blasts exposed to this drug combination in vivo could reflect induction of cell death, maturation, or a combination of the two. Whatever the mechanism responsible for synergistic antileukemic interactions between these agents, these considerations provide a strong rationale for exploring the activity of this combination regimen in patients with refractory/relapsed leukemia.

The results of the present Phase I study, in which bryostatin was given as a continuous 24-h infusion before and after four doses of HiDAC separated by a 5-day interval, demonstrate that administration of this combination regimen is feasible in patients with refractory acute leukemia, with a toxicity profile similar to that of other regimens used in the treatment of this disease. The MTD of bryostatin given in conjunction with 1.5 g/m² ara-C was determined to be 50 μg/m². At this dose level, myalgias represented the major DLT. This toxicity is similar to that observed in other single agent Phase I trials, in which bryostatin 1 was administered weekly for periods of 8 weeks, or weekly × 3 weeks every month (17–20). The mechanism responsible for bryostatin 1-mediated myalgias is uncertain but may be related to elaboration of cytokines such as tumor necrosis factor α (18). Whereas it is possible that circumvention of bryostatin 1-related myalgias could allow higher (and potentially more effective) doses to be administered, the possibility...
that such an intervention might antagonize the antileukemic activity of bryostatin 1 cannot be excluded. Other toxicities encountered included prolonged myelosuppression, which is difficult to interpret in patients with refractory leukemia, and hepatotoxicity. The latter two toxicities were observed in patients in whom the HiDAC dose had been increased to 3 g/m². In our earlier Phase I bryostatin 1 trial in patients with refractory solid tumors, hepatotoxicity was also noted at the higher bryostatin 1 doses (34). Given the fact that HiDAC is known to induce hepatitis (42), it is not surprising that hepatotoxicity precludes administering bryostatin 1 with high doses of ara-C. In any case, lack of clear evidence that a 3 g/m² dose is superior to a lower dose (i.e., 1 g/m²), at least in regard to overall survival (36), may render it unnecessary to attempt to combine bryostatin 1 with doses higher than the 1.5 g/m² dose initially used in this trial. Based on the present results, a bryostatin 1 dose of 50 µg/m² in conjunction with 1.5 g/m² of ara-C has been selected as the recommended Phase II dose for subsequent clinical evaluation. However, in view of the lack of a clear dose-response relationship between the bryostatin 1 level and clinical outcome, the possibility that a lower bryostatin 1 dose might prove more effective cannot be ruled out at this point.

The results of this Phase I trial, although preliminary, suggest that this regimen has activity in a cohort of heavily pretreated patients with refractory/refractory leukemia, with 4 CR observed among 25 evaluable patients and an additional CR obtained in 1 invaluable patient treated at the 3.0 g/m² ara-C level. Although treatment of relapsed leukemic patients with HiDAC alone has been associated with complete response rates of 20–30% (43), results in other series have been inferior i.e., as low as 12% (44). In this regard, retrospective analyses of the results of salvage regimens in patients with relapsed AML have revealed response rates as high as 50% in those with favorable prognostic features and as low as 5% in patients with adverse characteristics [e.g., relapse after multiple regimens, initial treatment failure, or early (<6 months) relapse, and so forth; Ref. 45]. While the relatively few patients in this trial did not permit stratification according to risk factors, the achievement of multiple CRs in several highly refractory patients, including those who had failed previous HiDAC or ABMT, suggest that this regimen has significant activity in relapsed acute leukemia, particularly in the setting of a Phase I trial. Thus, it is clear that a more definitive assessment of the activity of this regimen, in which drugs are administered at their MTD, will require evaluation of a larger group of patients in the context of a Phase II trial.

It is recognized that the design of this study did not permit direct comparisons to be made between the toxicity and antileukemic activity of the two drug sequences investigated i.e., bryostatin 1 →HiDAC and HiDAC→bryostatin 1. In the trial initially described by Capizzi et al. (35), in which an identical HiDAC schedule was used, l-asparaginase was given exclusively after each course of HiDAC based on preclinical evidence that protein synthesis inhibition after ara-C exposure maximized leukemic cell killing. In contrast, we found that previous (but not subsequent) administration of bryostatin 1 potentiated ara-C-induced lethality in cells (HL-60) insensitive to bryostatin 1-mediated maturation (22), whereas only subsequent bryostatin 1 treatment enhanced ara-C-induced apoptosis in leukemic cells (U937) partially sensitive to bryostatin 1-related differentiation (32). Whereas the conduct of two separate Phase I trials in which bryostatin 1 is given either before or after each course of HiDAC might allow the toxicity and activity of the two drug schedules to be compared, such an approach has the theoretical disadvantage of subjecting certain patients to a suboptimal drug sequence. In addition, blasts from individual patients may respond heterogeneously to bryostatin 1, which could allow a population of cells to escape the lethal effects of a particular drug sequence. Consequently, it was elected to incorporate both bryostatin 1/HiDAC schedules into the trial design in an effort to maximize leukemic cell killing and to optimize clinical outcome. However, a clearer definition of the sequence-dependent effects of bryostatin 1 and HiDAC in relation to host toxicity and antileukemic activity will require additional evaluation through the performance of separate clinical trials.

Chronic in vitro exposure of leukemic cells to bryostatin 1 results in PKC down-regulation, at least when bryostatin 1 is administered at concentrations ≥10 nM (22, 32). However, the effects of in vivo administration of bryostatin 1 on leukemic blast PKC activity was highly variable, analogous to results obtained in an earlier Phase I trial in which normal PBMCs were monitored (34). Such heterogeneous actions could reflect dose-dependent effects of bryostatin 1 on neoplastic cells, or, alternatively, variability in the responses of leukemic blasts to this agent (11). Unfortunately, the lack of a sufficiently sensitive pharmacokinetic assay (46) precludes relating effects of bryostatin 1 on blast PKC activity to plasma levels. In any case, attributable in part to the limited number of samples available for analysis, no clear relationship could be established between bryostatin 1 dose level and down-regulation of PKC activity in leukemic blasts, arguing against a dose threshold for this phenomenon. In addition, it was not feasible to assess PKC activity in blasts at the time of the second course of HiDAC, because significant cyto reduction had occurred at that time in all of the patients. Nevertheless, PKC activity was significantly reduced in 4 of 10 assayed specimens after the initial dose of bryostatin 1, demonstrating that in vivo administration of bryostatin 1 can down-regulate PKC activity in at least a subset of leukemic blasts. It remains to be established whether administration of bryostatin 1 at its MTD in a larger series of patients will consistently lead to PKC down-regulation in leukemic specimens, as observed in several in vitro studies (22, 32).

As in the case of PKC down-regulation, effects of in vivo administration of bryostatin 1 on the in vitro apoptotic response of leukemic blasts to ara-C was also variable, ranging from a modest reduction to no effect to an increase in 4 specimens. Moreover, no clear relationship emerged with respect to the effects of bryostatin 1 on leukemic blast PKC activity and their susceptibility to ara-C-induced apoptosis. Interestingly, in 3 of 4 of the latter samples, increases in cell death were largely attributable to in vivo responses to bryostatin 1 rather than to potentiation of ara-C-induced lethality. In fact, ex vivo responses of blasts to ara-C were marginal, and in all of the cases significantly less than those observed in continuously cultured cell lines such as HL-60 and U937 (22). This may reflect the relatively low S phase fraction of primary leukemic blasts (47), previous development of ara-C resistance (48), or a combination of these factors. In any event, based on this very limited sample
population, it cannot be concluded that previous ex vivo exposure to bryostatin 1 increases the susceptibility of primary blasts specimens to ara-C-related lethality, as observed previously in HL-60 cells in vitro. However, it remains possible that sequential in vivo exposure of such cells to ara-C followed by bryostatin 1, as occurred on days 9 and 10 of treatment, may result in enhanced lethality analogous to results obtained in U937 cells (32). Because blasts were no longer recoverable from the peripheral blood at this time, it was not feasible to test this hypothesis directly.

It is important to note that in vivo administration of bryostatin 1 alone did induce changes in primary leukemic blasts consistent with cell death, including increased uptake of 7-AAD and TUNEL-positivity, in a subset of patients. This indicates that plasma bryostatin 1 levels, despite being below concentrations detectable by current methods, are nevertheless capable of exerting lethal effects in at least some leukemic specimens. Finally, whereas apoptotic cells have been detected in the peripheral blood of patients receiving chemotherapy (49), the ability of the reticuloendothelial system to eliminate such cells is well described (50). Consequently, the possibility that the blasts available for these studies represent a subpopulation of cells that have escaped scavenging by the reticuloendothelial system must be considered in the interpretation of results.

In summary, the results of this trial demonstrate the feasibility of administering a 24-h infusion of bryostatin 1 before and after a course of HiDAC (1.5 g/m2 every 12 h on days 2 and 3, and 9 and 10) in patients with refractory/relapsed leukemia, and identify a bryostatin 1 dose of 50 μg/m2 as the MTD for this drug combination. In vivo administration of bryostatin 1 exerted variable effects on leukemic blast PKC activity as well as the extent of apoptosis detected in cells exposed to ara-C ex vivo. Significantly, ex vivo administration of bryostatin 1 alone exerted cytotoxic activity toward leukemic blasts in a subset of patients. Finally, the preliminary results of this study suggests that bryostatin 1 may have biological activity in AML, at least in a subset of patients. A clearer assessment of the antileukemic activity of the combination of HiDAC and bryostatin 1 will require evaluation of a larger number of patients treated in a uniform manner i.e., at the bryostatin 1 MTD. Such an analysis may also permit more definitive conclusions to be drawn regarding correlations between the effects of bryostatin 1 on PKC activity and ara-C-related apoptosis, as well as possible relationships between either of these determinants and clinical outcome. To address these and related questions, a multi-institutional Phase II trial combining HiDAC and bryostatin 1 for patients with refractory/relapsed AML has been initiated recently.

REFERENCES


Phase I Trial and Correlative Laboratory Studies of Bryostatin 1 (NSC 339555) and High-Dose 1-B-d-Arabinofuranosylcytosine in Patients with Refractory Acute Leukemia


Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/8/7/2123

This article cites 49 articles, 23 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/8/7/2123.full#ref-list-1

This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/8/7/2123.full#related-urls

Sign up to receive free email-alerts related to this article or journal.
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/8/7/2123.
Click on “Request Permissions” which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.