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Differentiation therapy in poor risk myeloid malignancies: Results of a dose finding study of the combination bryostatin-1 and GM-CSF

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Abstract

Purpose—Pharmacologic differentiating agents have had relatively limited clinical success outside of the use of ATRA in acute promyelocytic leukemia and DNA methyltransferase inhibitors in myelodysplastic syndromes. The differentiating effects of such agents can be enhanced in combination with lineage-specific growth factors. We developed a dose finding trial to assess toxicity, differentiating activity, and clinical impact of the combination of bryostatin-1 and GM-CSF.

Experimental design—Patients with poor risk myeloid malignancies were eligible to enroll in a dose finding study of continuous infusion bryostatin-1 combined with a fixed dose of daily GM-CSF. Toxicities were graded per NCI CTC version 2.0 and pharmacokinetic and correlative study samples were obtained to assess the combination's clinical and biologic differentiating effects.

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Conflict of interest statement

There are no conflicts of interest to report.

Contributions. BDS: designed clinical trial, performed research, collected, analyzed, and interpreted data, wrote the manuscript; RJJ: designed clinical trial, performed research, wrote the manuscript; EC, JK and EDW: analyzed and interpreted data, performed statistical analysis, wrote the manuscript; JEK and SDG: contributed patients, performed research; MV and BM: contributed vital analytical tools, performed research; SDB: designed clinical trial, contributed analytical tools; MZ: contributed analytical tools, analyzed, and interpreted data; SP: designed clinical trial, analyzed and interpreted data, and performed statistical analysis; ZZ: performed statistical analysis; GB: contributed vital analytical tools; RAB: designed research, contributed patients; AM: designed clinical trial; MAR: contributed vital analytical tools, performed research, collected, analyzed, and interpreted data, wrote the manuscript and WHM: designed research, contributed vital analytical tools, performed research, collected, analyzed, and interpreted data, wrote the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2010.06.001.

Results—Thirty-two patients were treated with the combination therapy and the dose determined to be most suitable for study in a larger trial was continuous infusion bryostatin-1 at 16 $\mu\text{g}/\text{m}^2$ for 14 days and subcutaneous GM-CSF at 125 $\mu\text{g}/\text{m}^2$ daily for 14 days every 28 days. Arthralgias and myalgias limited further dose escalation. Clinically, the combination impacted differentiation with improvement of absolute neutrophil counts ($p = 0.0001$) in the majority of patients. Interestingly, there were two objective clinical responses, including a CR after a single cycle. Both the bryostatin-1 plasma concentrations and the correlative studies supported biologic activity of the combination at the doses where clinical responses were observed.

Conclusions—Combining growth factors with pharmacologic differentiating agents may increase their clinical effectiveness and further studies should focus on such combinations.

Keywords

Leukemia; Differentiation therapy; Myelodysplastic syndrome; Growth factors; Bryostatin; Drug resistance

1. Introduction

The macrocyclic lactone bryostatin-1 has been studied as an anti-cancer agent for nearly 3 decades [1]. Bryostatin-1 acts as a partial agonist of protein kinase C (PKC) [2,3], but the exact mechanisms accounting for its anti-cancer effects are unclear. Previous studies have found that bryostatin-1 has differential effects on malignant and normal hematopoiesis. It may enhance normal hematopoiesis [4–8]. In contrast, bryostatin-1 inhibits the clonogenic growth of human AML cell lines by inducing cell cycle arrest and phenotypic differentiation [9,10]. We previously found that the addition of myeloid growth factors markedly enhanced leukemic differentiation. Moreover, neutralizing antibodies directed against myeloid growth factors blocked the differentiating potential of bryostatin-1 and suggested that growth factors are necessary for its full anti-leukemic activity [11,12].

As single agents, myeloid growth factors may primarily enhance leukemic proliferation. However, in combination with bryostatin-1, the predominant effects appear to be induction of malignant cell differentiation while sparing normal hematopoiesis. These dual actions are particularly attractive for translation in myeloid malignancies, such as myelodysplastic syndrome and acute myeloid leukemia that are characterized by ineffective or blocked differentiation and bone marrow failure. Furthermore, leukemic stem cells are thought to contain the self-renewal potential of the malignant clone while retaining the capacity to undergo differentiation. Importantly, self-renewing potential is lost with the initiation of differentiation and forced terminal differentiation may lead to the elimination of this malignant population [13,14]. Based on *in vitro* work supporting the differentiating impact of the combination [11,12], we undertook a dose escalation trial of continuous infusion bryostatin-1 and daily GM-CSF to determine the maximum tolerated dose (MTD), assess the combination's toxicity profile, and establish pharmacokinetics (PK) of bryostatin-1. We also assessed the combination's biological and clinical activities in relapsed and refractory myeloid malignancies.

2. Patients/methods

Adult patients over the age of 18 with a diagnosis of either relapsed or refractory acute myeloid leukemia, poor risk myelodysplastic syndrome, accelerated phase or blast crisis chronic myeloid leukemia, or progressive paroxysmal nocturnal hemoglobinuria (PNH) were considered eligible provided they were not immediate candidates for potentially curative allogeneic stem cell transplant. Patients were required to have Eastern Cooperative

Oncology Group (ECOG) performance status 0, 1, or 2, stable bone marrow function for the 10 days prior to enrollment, and otherwise normal hepatic and renal function. Patients with elevated white blood counts (WBCs) at the time of study enrollment were permitted treatment with oral hydroxyurea to maintain $\text{WBC} < 30,000/\mu\text{L}$ for up to 7 days into the initial cycle of combination therapy. Patients with persistently elevated WBC at the completion of the allotted hydroxyurea were removed from the study. Protocol and consent form were approved by the Johns Hopkins School of Medicine Institutional Review Board.

2.1. Treatment plan

Patients were initiated on the combination of bryostatin-1 and GM-CSF for either 14 or 21 consecutive days of treatment in each 28-day cycle. Bryostatin-1 (supplied by NCI Cancer Therapy Evaluation Program) was administered intravenously by continuous pump infusion based on previous data suggesting that continuous exposure to bryostatin-1 is required to maximize the drug's antiproliferative effect *in vitro* against leukemias and lymphomas [15]. GM-CSF was self-administered subcutaneously each day (either for 14 or 21 days) at a set dose of $125 \mu\text{g}/\text{m}^2/\text{day}$. Bryostatin-1 starting dose was recommended at $6 \mu\text{g}/\text{m}^2 \times 14$ days (total dose $84 \mu\text{g}/\text{m}^2$) and was escalated in $2 \mu\text{g}/\text{m}^2/\text{day}$ increments through $18 \mu\text{g}/\text{m}^2 \times 21$ days (total dose $252 \mu\text{g}/\text{m}^2$). The escalation was determined based on the modified continual reassessment method (CRM) [16,17]. The CRM is not strictly a dose escalation regimen, but is a Bayesian dose finding method designed to select a dose level with a pre-specified probability of dose-limiting toxicity. The maximally tolerated dose was operationally defined as the dose level that yielded dose-limiting toxicities in 30% of patients. Two completed treatment cycles without dose-limiting toxicities were required in at least 2 patients on the active dosing level before to proceeding to the next dose level. Patients with improvement of disease after the initial 2 cycles of therapy, defined as a complete or partial response, could continue on their current dosing level for up to a total of 12 cycles (1 year) provided there was no evidence of disease progression and no dose-limiting toxicity secondary to protocol therapy. Inpatient dose escalation was permitted in patients with stable disease after their initial 2 cycles of therapy provided they had no evidence of dose-limiting toxicities related to protocol therapy. Evidence of improvement at the higher dosing or ongoing bone marrow stability allowed patients to continue on additional cycles at the new dose as described above. Dose modification of the GM-CSF was permitted in patients where the combination therapy resulted in increased peripheral WBC. GM-CSF was held for $\text{WBC} > 30,000/\mu\text{L}$ and was restarted after 1 week at 75% of the original dose provided the WBC fell to $< 20,000/\mu\text{L}$. An additional dose reduction of 25% was made if $\text{WBC} > 30,000/\mu\text{L}$ a second time and patients were removed from study for a $\text{WBC} > 30,000/\mu\text{L}$ following the two described dose reductions.

2.2. Response assessment

Baseline assessments were conducted within 1 week prior to entry into the study and included physical examinations, ECOG performance status, routine laboratory evaluations (complete blood count (CBC) with differential, platelets, biochemistry, clotting times, and urinalysis), and bone marrow aspirate or biopsy with cytogenetic analysis. Blood counts, including white blood cell (WBC), absolute neutrophil count (ANC), hematocrit (HCT), and platelets (PLT), were followed twice a week for the first 2 cycles of therapy and at least weekly thereafter. Packed red blood cell and platelet transfusions were also monitored weekly. Indications for transfusions were highly individualized but in general, the target HCT was $> 25\text{--}28\%$ and the target PLT level was $> 10,000/\mu\text{L}$. In patients with fever, active infections, or active bleeding, the target PLT level was increased to $> 20,000/\mu\text{L}$.

Overall clinical responses were assessed by repeat bone marrow aspirate and biopsy at the completion of 2 cycles of therapy. Peripheral blood response assessments were ongoing

throughout the trial. Patients who were taken off-study due to progression or toxicity underwent an “off-study” bone marrow to assess response. Clinical responses for AML and MDS were measured according to International Working Group definitions of complete response (CR), partial response (PR), stable disease (SD), hematologic improvement, and progressive disease (PD) [18,19]. Patients were also evaluated for cytogenetic responses defined as either major (no evidence of initial cytogenetics) or minor (>50% reduction). Finally, response criteria for paroxysmal nocturnal hemoglobinuria were based on improvement or resolution of clinical symptoms, normalization of peripheral blood counts and a decrease in the granulocyte cell population lacking CD59 to <5% (CR) or <25% (PR) of the total granulocytes.

2.3. Determination of DLT, MTD and stopping rules

All patients filled out medication and side effect/toxicity diaries and were examined by a study team member twice weekly for each week of their initial 2 cycles of therapy and at least once weekly thereafter. Toxicities were graded according to the National Cancer Institute Common Toxicity Criteria, Version 2.0. Dose-limiting toxicities (DLTs) were defined as grade ≥ 3 , non-hematologic toxicity that did not resolve upon discontinuation of the combination therapy prior to the start of their next planned cycle or grade IV bone marrow cellularity (i.e., aplasia or >6 weeks to recovery of normal marrow cellularity). DLTs were determined from toxicities seen in all cycles. Patients requiring cessation of the treatment were monitored for recovery and resolution of their symptoms. Patients did not receive further treatment on the study if their therapy was arrested for >4 weeks. The MTD was defined as the dose at which the CRM estimated that approximately 30% of patients would experience DLT.

2.4. Pharmacokinetic sampling, analytic assay and data analysis

Initially, blood samples were collected in heparinized tubes for pharmacokinetic analysis before bryostatin-1 administration, at 1 h, 24 h, 8 days after the start of the infusion, and then weekly until the end of the infusion (day 15 or day 22 depending on the dose level). Previous descriptions of bryostatin-1 pharmacokinetics were based on indirect analytical methods [20–22] as direct analytical methods using HPLC-based techniques lacked the necessary sensitivity to quantitate bryostatin-1 in human plasma [23–25]. During the conduct of this study, we developed and validated the first LC/MS/MS method sensitive enough to elucidate and quantify bryostatin-1 concentrations in plasma [26]. Subsequently, additional samples were obtained 5 min prior to the end of the infusion and then at 0.25, 0.50, 0.75, 1, 2, 4, and 6 h after the end of the infusion. The samples were processed within 30 min by centrifugation at $3000 \times g$ for 10 min at 4 °C. The resulting plasma was separated and stored at –70 °C until analysis. Bryostatin-1 plasma concentrations were determined over the range of 50–2000 pg/mL using a validated LC/MS/MS analytical assay [26]. Individual concentration-time data were analyzed using noncompartmental methods using WinNonlin Professional version 5.0 (Pharsight, Mountain View, CA) [27]. Steady-state concentration (C_{ss}) was the concentration obtained prior to the end of the infusion. Bryostatin-1 pharmacokinetic parameters were summarized using descriptive statistics.

2.5. Laboratory correlates

An *in vitro* study was conducted to measure the suppression of clonogenic recovery of the cell line NB4 by patient serum as a measure of the bioactivity of bryostatin-1. A paired analysis was conducted in 9 patient samples with plasma drawn prior to and during the course of the continuous infusion of bryostatin-1 treatment. The human APL cell line NB4 [28] was maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL, Rockville, MD), 50 U/mL penicillin, 50 µg/mL streptomycin, and 2 mM L-glutamine. For clonogenic assays cells (500/mL) were resuspended in 1.0 mL of

1.2% methylcellulose containing 30% FBS, 1% BSA, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, and 10% patient plasma serum or normal human serum (Sigma, St. Louis, MO) containing various concentrations of bryostatin-1 (0.1–100 nM; National Cancer Institute, Bethesda, MD) [12]. Samples were plated in quadruplicate onto 35 mm² tissue culture dishes and incubated in a humidified atmosphere at 37 °C and 5% CO₂. Colonies consisting of >40 cells were counted using an inverted microscope at 7–10 days.

3. Results

3.1. Patient characteristics

A total of 32 patients with poor risk, relapsed or refractory myeloid malignancies were treated between April 2001 and February 2005 with a total of 70 (52 complete) cycles of the combination bryostatin-1 and GM-CSF (Table 1). The median age of the patients was 65 (range 23–75) years and 23 (72%) patients were male. The 17 AML patients included 8 with AML arising from MDS or with underlying multilineage dysplasia, 3 with therapy-related AML, and 6 with relapsed or refractory *de novo* AML. Of the 14 MDS patients, 11 had evidence of poor risk disease based on the number of lineages affected, transfusion requirements, cytogenetics, or IPSS, when available. The remaining 3 subjects' had disease progression despite growth factor and routine supportive care.

3.2. Combination therapy toxicities and determination of maximally tolerated dose

The toxicities of the combination of bryostatin-1 and GM-CSF are listed according to dose level in Table 2. All subjects receiving any dose of either of the two agents were considered evaluable for toxicities. As predicted from previous phases I and II studies of bryostatin-1, grade 3/4 myalgias and arthralgias were dose limiting for the combination. Muscle and generalized weakness were also common in our patients; however, based on their age and progressive bone marrow failure, it was difficult to determine the specific role of the drug combination in their constitutional symptoms. Neutropenic fevers ($n = 7$ cycles), neutropenic infections ($n = 2$ cycles), and non-neutropenic infection ($n = 1$ cycle) occurred as expected in this advanced group of patients and were not considered dose-limiting. Other notable toxicities included mild recurrent dyspnea ($n = 8$ cycles), cough ($n = 1$ cycle), and frequent urination ($n = 5$ cycles). Although the majority of subjects treated carried the diagnosis of acute leukemia, expected toxicities of the GM-CSF were minimal with only two patients requiring a 25% dose reduction of GM-CSF for WBC > 30,000/ μ L. Fig. 1 shows estimated dose-limiting toxicity (DLT) probabilities based on observed data (see Table 2) and CRM model fit. Based on Fig. 1, the MTD is approximated at 16 μ g/m²/day, with an estimated DLT target of around 0.33. The CRM model selection of 16 μ g/m²/day of bryostatin-1 given as a continuous infusion in combination with 125 μ g/m²/day of GM-CSF subcutaneously for 14 days of a 28-day cycle as the dose suitable for larger studies reflects the accumulation of clinical experience from all doses (a feature of the CRM model), not simply the clinical experience at any one dose, and the CRM's fitting and smoothing properties.

3.3. Clinical response

A total of 70 cycles of the combination therapy were given to the 32 patients enrolled with 27 (85%) patients completing at least 1 cycle and 20 of the 32 patients completing 2 or more cycles. Five patients (16%) failed to complete their initial cycle of which 2 subjects withdrew their consent (1 secondary to recurrent tumor fevers and 1 due to chronic fatigue related to disease progression) and elected hospice care during their first week of therapy and were not considered evaluable. Of the remaining 30 subjects, 9 discontinued the trial after 1 cycle due to patient preference ($n = 3$), disease progression ($n = 5$), or failure of resolution of previous grade 3 or above toxicities ($n = 1$).

The 20 patients completing at least 2 cycles were evaluable for protocol-specified response assessment after 2 cycles as per the original study design. Of these, 1 patient with MDS had a marrow CR and became transfusion independent, 11 had SD, and 8 had PD. One patient achieved a complete remission following a single cycle of therapy at the highest total bryostatin-1 dosing level, but unfortunately, toxicity prevented additional treatment. The impact of the combination on peripheral blood counts was noted as early as the first cycle of therapy with half of the cycles ($n = 33$) being associated with a measurable hematologic improvement (Table 3). Mean differences in blood counts and transfusion requirements between pre- and post-initiation of combination therapy for cycle #1 were compared using a generalized estimation equation (GEE) model to account for correlated measurements within same subjects (using an exchangeable correlation structure). Statistically significant increases in weighted means were observed between pre- and post-treatment values for both WBC and ANC (Fig. 2). Unfortunately, this early improvement in counts did not immediately translate into lower transfusion requirements for red blood cells ($p = 0.60$) or platelets ($p = 0.10$). Nonetheless, clinically important responses were observed in this poor risk group of patients following as little as 1 cycle of therapy, as described below.

3.4. Clinical responses scenarios

Patient 1 was a 60-year old male diagnosed with MDS, RARS, in 09/2003. His disease was characterized by a hypercellular marrow with 5% blasts by flow cytometry, 46 XY del (20)(q11.2q13.2) karyotype in 20/20 metaphases and overall IPSS of 1.5. The patient was referred for further evaluation based on red blood cell transfusion requirements of 2–3 units every 1–2 weeks and a marked impact on quality of life. He was treated with bryostatin-1 at $20 \mu\text{g}/\text{m}^2 \times 14$ days. Because of significant myalgias and arthralgias, the second cycle was given at a one dose reduction to $18 \mu\text{g}/\text{m}^2 \times 14$ days. Unfortunately, he was only able to tolerated 9 days of the second cycle due to recurrent toxicities. During the active phase of his treatment, his peripheral counts fluctuated with lineage responses in both the neutrophils and platelets. However, over the following 2 months, the patient became transfusion independent. He remained free of symptoms for 18 months post-therapy with a neutrophil count of greater than $2000/\mu\text{L}$ and platelet count greater than $100,000/\mu\text{L}$. A repeat bone marrow one year post-therapy showed normal to hypocellularity, minimal evidence of dysplasia and an improved cytogenetic profile with his original del 20q now comprising only 30% metaphases. Repeat studies at 18 months (12/2008) showed a cellular to hypocellular marrow with continued decrease in the original del 20q clone now found in 15% of the metaphases. He remains alive with transfusion dependent MDS.

Patient 2 was a 73-year old male diagnosed with *de novo* AML in January 2004 with neutropenia, mild thrombocytopenia, and peripheral blasts. His bone marrow aspirate and biopsy was hypercellular with greater than 50% of the cellularity being consistent with myeloblasts by phenotype. His AML had normal cytogenetics (46XY). He completed cycle 1 of combination with bryostatin-1 at $16 \mu\text{g}/\text{m}^2 \times 21$ days but could not continue due to slow resolution of grade 3 myalgias and arthralgias. Like patient 1, he experienced lineage responses in neutrophils during his infusional combination therapy, his counts continued to improve, and he became transfusion independent after therapy was discontinued. A repeat bone marrow aspirate and biopsy at 3 months off therapy was hypocellular and showed a complete morphologic remission with no evidence of residual leukemia. He remained completely free of transfusions for 10 months at which time a repeat bone marrow was performed due to falling peripheral blood counts and confirmed the re-emergence of his original blast population.

3.5. Pharmacokinetic studies

Plasma pharmacokinetic studies of bryostatin-1 were completed on 19 patients over a total of 30 assessment periods at infusional dose levels greater than or equal to $12 \mu\text{g}/\text{m}^2$. Patients receiving infusional bryostatin-1 at doses below $12 \mu\text{g}/\text{m}^2$ were not studied as their plasma drug concentrations were below the limit of quantification ($<50 \text{ pg}/\text{mL}$). Samples for assessment of steady-state concentration (C_{ss}) were obtained prior to the end of the infusion on 23 of 30 assessment periods and were $<50 \text{ pg}/\text{mL}$ in 6 instances (see Fig. 3A). During the first cycle, the C_{ss} value varied up to 2.9-fold at $18 \mu\text{g}/\text{m}^2/\text{day}$ while this variability increased to 3.4-fold with subsequent administration. Complete pharmacokinetic profiles were obtained in 7 patients for 8 assessment periods. Although several profiles ($n = 5$) had concentration versus time profiles expected for a typical continuous IV infusion (i.e., predictable increases in plasma concentrations up to the end of the infusion with smooth decreases after drug discontinuation), a significant number of profiles ($n = 3$) demonstrated intermittent spikes during the infusions or post-infusional peaks several hours after the discontinuation of bryostatin-1 (see Fig. 3B–D). During the first cycle, the $t^{1/2}$ value was 3.36 or 3.91 h ($n = 2$) while it increased to $11.19 \pm 2.50 \text{ h}$ ($n = 3$) with subsequent administration. Clearance values were highly variable ($23.1 \pm 17.9 \text{ L}/\text{h}$; $n = 5$; 77.5% CV).

3.6. Laboratory correlates

The paired analysis measuring the suppression of clonogenic recovery of NB4 cells by patient serum obtained prior to and during treatment was compared to a standard curve of known concentrations of bryostatin-1 (see Supplement, Fig. S1). These results were used to extrapolate drug levels in patients for whom pharmacokinetic analysis was not sensitive enough to quantify plasma concentrations of bryostatin-1. As expected, a dose-dependent inhibition of colony formation was observed with the most significant reduction in colony growth was associated with using serum collected from patients receiving $20 \mu\text{g}/\text{m}^2/\text{day}$ of bryostatin-1. These results are consistent with the notion that biologically relevant doses of bryostatin-1 were achieved under the study protocol.

4. Discussion

A major challenge in the development of anti-cancer strategies that induce differentiation and potentially target cancer stem cells is accurately evaluating and classifying the clinical activity. Using standard AML or MDS response criteria is problematic as they largely reflect changes in total tumor bulk following cytoreductive therapy. Paradoxically, increased tumor burden due to an expansion of mature tumor cell compartments, such as ATRA syndrome in APL, may be an early indicator of successful differentiation activity. In our current study, the mean weighted WBC and ANC, but not the peripheral blasts, significantly increased during the treatment period. It is unclear whether this increase was due to differentiation of malignant progenitors or improvement of normal hematopoiesis. Nonetheless, this quantitative increase was also functional as it appeared to limit the infectious complications of neutropenia since none of the study participants treated in the highest 3 dosing cohorts ($18 \mu\text{g}/\text{m}^2 \times 14 \text{ days}$, $20 \mu\text{g}/\text{m}^2 \times 14 \text{ days}$, $16 \mu\text{g}/\text{m}^2 \times 21 \text{ days}$) experienced febrile episodes over 18 cumulative cycles. Importantly, improvements in ANC and febrile episodes were not limited to patients with objective responses in bone marrow blasts or changes in transfusion needs suggesting that bryostatin-1 may have clinical activity at doses that do not suppress normal hematopoiesis [15].

It is also possible that the improvement in blood counts, namely neutrophils, was due to activity of GM-CSF alone rather than the combination. However, based on known similar effects on both normal and malignant cells, one might expect to see a concurrent increase in peripheral blasts along with the higher total white cell count. We did not observe these

effects even in patients with active AML at the time of treatment suggesting that the combination primarily impacts normal myeloid growth and maturation either through the inhibition of the malignant clone or direct enhancement of normal hematopoiesis.

The kinetics of clinical responses may also differ between traditional cytotoxic agents and those targeting differentiation pathways. Limiting the self-renewal capacity of leukemic stem cells would likely not produce an immediate or dramatic response, but rather a more delayed and gradual one as the production of new tumor cells was inhibited. Although the anti-leukemic effects of bryostatin-1 and GM-CSF were modest during treatment, two of the patients treated at the highest doses of bryostatin-1 experienced durable improvements in both leukemic blast counts and normal peripheral blood counts. Interestingly, these improvements were not immediate but gradual in nature following the discontinuation of the treatment due to toxicity. Importantly, these responses were prolonged with the one CR lasting for approximately one year and the second patient free of transfusions for 18 months. Although far from definitive, these results suggest that differentiation targeted therapies may target leukemia stem cells in a clinically meaningful fashion.

The pharmacology of bryostatin-1 administered by continuous infusion is complex and limited by availability of sensitive analytical techniques [23–25]. While our analytical method could measure bryostatin-1 levels as low as 50 pg/mL [26], a more sensitive analytical method should be developed in order to understand this agent's clinical pharmacology. We were able to assess whether biologically relevant levels of drug were reached in patients through an *in vitro* correlative study using patient plasma pre- and during treatment on a cell line known to be sensitive to bryostatin-1. Even though doses of bryostatin-1 under 12 $\mu\text{g}/\text{m}^2$ were not measurable in patient plasma samples with LC/MS/MS, the correlative assays demonstrated that drug levels in the patient plasma samples could suppress cell growth. This clonogenic growth suppression appeared to occur in a dose–response fashion in samples studied. Using a control dose–response curve, drug levels were extrapolated by quantifying clonogenic recovery and showed that continuous infusion of bryostatin-1 was effective at suppressing 40% of colony growth at doses equal to or greater than 16 $\mu\text{g}/\text{m}^2$. Such studies are helpful in providing secondary evidence of drug activity and may also reflect the impact of biologic agents on residual and small populations of disease. In addition to these studies, efforts to quantify leukemia stem cells serially should be undertaken and further developed to be incorporated more broadly into differentiation based clinical trials.

Unfortunately, there were significant toxicities seen with this combination. Consistent with previous clinical trials involving bryostatin-1, myalgias and arthralgias were the predominant dose-dependent adverse events that precluded participants from tolerating maximal dose escalation [29,30]. However, the addition of growth factor did not appear to worsen or lessen these expected bryostatin-1 toxicities. Though the toxicities of bryostatin-1 limit its further development, this combination serves as a template for new studies combining growth factors with pharmacologic differentiation agents that offer better side effect profiles. Future studies will benefit from efforts to integrate correlative studies, such as the serial measurements of leukemic stem cells, and together may determine whether approaches targeting differentiation ultimately inhibit these clonogenic cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

The diamonds are based on the observed data (see Table 2, DLT proportion among patients) while the line denotes the CRM model fit. The Y-axis represents the estimated probability of toxicity and the X-axis dose level of bryostatin-1 ($\mu\text{g}/\text{m}^2/\text{day}$) studied.

Fig. 2.

Log transformed weighted individual blood counts for patients on the combination of bryostatin-1 + GM-CSF during cycle 1 ($n = 28$). Analysis using a generalize estimate equation showed improvement of both the white blood cell count ($p < 0.0001$) and the absolute neutrophil count ($p < 0.0001$) while changes in the hematocrit and platelet counts did not reach statistical significance. The length of the box is the inter-quartile range and represents the middle 50% of the data. The horizontal line inside the box shows the median. The lower and upper hinges of the box represent the 25th and 75th percentiles, respectively. The dashed lines extend from the box to the upper and lower 1.5 inter-quartile values from the upper and lower hinges. The open circles far out represent the outliers. The solid circles represent the actual log transformed blood count values.

Fig. 3.

(A) The steady-state concentrations as shown with the opened circles ($\circ$) as the first cycle C_{ss} values while the closed circles ($\bullet$) are from subsequent cycles. The Y-axis represents the concentration at steady state in pg/mL and the X-axis dose level of bryostatin-1 studied.

Samples <50 pg/mL were plotted for informational purposes at 0 pg/mL. (B–D)

Representative concentration versus time profiles following bryostatin-1 infusion: (B) 336-h infusion of bryostatin-1 $20 \mu\text{g}/\text{m}^2/\text{day}$; (C) 405-h infusion of bryostatin-1 $16 \mu\text{g}/\text{m}^2/\text{day}$; (D) 502-h infusion of bryostatin-1 $16 \mu\text{g}/\text{m}^2/\text{day}$. The Y-axis is the concentration of bryostatin in pg/mL on log scale, the X-axis is time in hours, and the solid line at 50 pg/mL represents the lower limit of quantitation for the assay.

Table 1

Patient characteristics.

Patient characteristics: <i>n</i> = 32		
Age (years)	Median age = 65 (range: 23–75) years	
Sex	Male <i>n</i> = 23 (72%)	Female <i>n</i> = 9 (28%)
Diagnosis	WHO classifications:	Cytogenetics:
	AML: <i>n</i> = 17 (53%)	AML:
	AML/MDS or AML with MLD: 8	Complex: 7
	t-AML: 3	Normal: 3
	relapsed/refractory <i>de novo</i> AML: 6	Unknown: 3
		Del 11q21: 1
	MDS: <i>n</i> = 14 (44%)	Trisomy 21: 1
	RAEB-1: 5	Deletion 7q22: 1
	RAEB-2: 3	Del 11q14: 1
	RCMD: 2	
	RAEBt: 1	MDS:
	RARS: 1	Complex: 6
	MDS-NOS: 2	Normal: 2
		Unknown: 2
	PNH: <i>n</i> = 1 (3%)	Del 20q: 3
		Trisomy 8: 1
		PNH: normal: 1
IPSS: <i>n</i> = 12 MDS pts*	Low: 0	
	Int-1: 4	
	Int-2: 7	
	High: 1	

* The IPSS data was only available for 12 out of the 14 patients with MDS. Cyto-genetics at diagnosis were not available for 2 MDS patients.

Table 2

Toxicities suspected related to bryostatatin-1.

Bryostatatin-1 dose level	Bryostatatin-1 dose (total)	Patients at dose level	Cycles per dose level (total)	DLT proportion among patients	Cycles with myalgia or arthralgia (grade 3/4)	Cycles with weakness (grade 3/4)	Other notable adverse events (grades 1 and 2)
6 $\mu\text{g}/\text{m}^2 \times 14$ days	84 $\mu\text{g}/\text{m}^2$	3	8	0	0	0	Dyspnea (1) Hypoxia(1) Infection – cellulitis(1) Syncope(1)
8 $\mu\text{g}/\text{m}^2 \times 14$ days	112 $\mu\text{g}/\text{m}^2$	3	8	0	0	0	Dyspnea (2) Febrile neutropenia (3)
10 $\mu\text{g}/\text{m}^2 \times 14$ days	140 $\mu\text{g}/\text{m}^2$	7	13	0.285	0	2	Abdominal pain (1) Anorexia (1) Dizziness (1) Dysphagia/stomatitis (3) Dyspnea (6) Erythema multiforme (1) Febrile neutropenia (3) Hemolysis (3) Hyperglycemia (4) Hypokalemia (4) Hypotension (1) Nausea (1) Tachycardia (1) Insomnia (1) Infection – catheter (1) Cough (1) Elevated lipase (1) Febrile neutropenia (1) Mental confusion (1) Voice changes (1) Febrile neutropenia (1) Ataxia (1)
12 $\mu\text{g}/\text{m}^2 \times 14$ days	168 $\mu\text{g}/\text{m}^2$	5	7	0	0	1	
14 $\mu\text{g}/\text{m}^2 \times 14$ days	196 $\mu\text{g}/\text{m}^2$	6	12	0.167	1	1	
16 $\mu\text{g}/\text{m}^2 \times 14$ days	224 $\mu\text{g}/\text{m}^2$	2	4	0	0	0	
18 $\mu\text{g}/\text{m}^2 \times 14$ days	252 $\mu\text{g}/\text{m}^2$	6	10	0.500	7	8	

Bryostatatin-1 dose level	Bryostatatin-1 dose (total)	Patients at dose level	Cycles per dose level (total)	DLT proportion among patients	Cycles with myalgia or arthralgia (grade 3/4)	Cycles with weakness (grade 3/4)	Other notable adverse events (grades 1 and 2)
20 $\mu\text{g}/\text{m}^2 \times 14$ days	280 $\mu\text{g}/\text{m}^2$	4	4	1	1	4	Hyperuricemia (1)
							Myositis (1)
							Infection – bladder (1)
							Polyuria (4)
							Thrombosis (1)
							Dyspnea (1)
							Polyuria (1)
16 $\mu\text{g}/\text{m}^2 \times 21$ days	336 $\mu\text{g}/\text{m}^2$	3	4	1	2	3	Hyponatremia (3)
							Hyperuricemia (3)
							Anorexia (1)
							Elevated ALT (2)
							Elevated AST (1)
							Polyuria (1)
							Nausea (1)
							Insomnia (2)

Table 3

Bryostatins-1 dose levels and observed clinical responses.

Dose level	Cycles at dose level (# of Pts)	Non-evaluable	Clinical response				Hematologic improvement	
			CR	PR	SD	PD	Yes	No
6 µg/m ² × 14 days	8 (3)	0	0	0	6	2	6	2
8 µg/m ² × 14 days	8 (3)	0	0	0	7	1	5	3
10 µg/m ² × 14 days	13 (7)	1	0	0	4	8	4	8
12 µg/m ² × 14 days	7 (5)	1	0	0	4	2	4	2
14 µg/m ² × 14 days	12 (6)	1	0	0	7	4	2	9
16 µg/m ² × 14 days	4 (2)	0	0	0	1	3	0	4
18 µg/m ² × 14 days	10 (6)	1	0	1	8	0	6	3
20 µg/m ² × 14 days	4 (4)	0	0	0	4	0	3	1
16 µg/m ² × 21 days	4 (3)	0	1	0	1	2	3	1

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.