Successful Treatment of Murine Melanoma with Bryostatin 1


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ABSTRACT

Bryostatins are a novel class of protein kinase C activators which were isolated from the marine bryozoan Bugula neritina and found to possess both antineoplastic and immunoenhancing properties. In this report, we examined the relationship between the in vivo and in vitro antineoplastic effects of bryostatin 1. The in vivo antitumor activity of bryostatin 1 was demonstrated in a B16 melanoma pulmonary metastases model, in which treatment of tumor-bearing C57BL/6 mice with 5 days of bryostatin 1 resulted in a significant reduction in the number of lung nodules (control, 87; bryostatin, 7). There was a clear dose-response effect, with the optimal antimalanoma dose being 100 μg/kg/day, but even low doses of bryostatin 1 of 1 μg/kg/day resulted in a 53% reduction in the number of metastases. Although bryostatin 1 shares many biological properties with the phorbol esters, parallel treatment with 12-O-tetradecanoyl 13-phorbol acetate was ineffective against B16 melanoma in vivo. Using a clonogenic assay, bryostatin 1 was found to have a direct antiproliferative effect against B16 melanoma. This inhibition occurred at relatively high bryostatin 1 concentrations (10^{-4} M), in comparison with a sensitive cell line REH (10^{-4} M). Treatment of mice with bryostatin 1 or preincubation of normal spleen cells with bryostatin 1 failed to enhance nonspecific cell-mediated cytotoxicity against B16 melanoma in vitro. Moreover, bryostatin 1 was found to inhibit both natural killer cell activity and interleukin 2 generation of lymphokine-activated killer cells. Thus, a role for an in vivo immune enhancement mechanism as the basis for the antimalanoma activity observed with bryostatin 1 cannot be invoked from these experiments. These findings indicate that bryostatin 1 may act directly on the B16 melanoma pulmonary metastases. The precise mechanism whereby bryostatin exerts its antimalanoma effects remains unclear.

INTRODUCTION

Although major advances have been made in the treatment of many malignancies, malignant melanoma remains refractory to current therapies. New approaches are needed to treat melanoma and other resistant cancers. Bryostatin 1 belongs to a newly discovered family of macrocyclic lactones isolated from the marine bryozoan Bugula neritina which possess diverse biological properties. Bryostatins have documented in vitro antineoplastic properties against a number of cancer cell lines, including melanoma, renal cell, and leukemia (1). In addition, prolongation of survival in bryostatin 1-treated animals has been reported in a murine P388 lymphocytic leukemia system and in an ovarian sarcoma model (2). Other biological properties of bryostatins include potent immunomodulatory effects, including activation of T-cells and augmentation of neutrophil and monocyte tumor cytotoxicity, and stimulation of bone marrow progenitor cells (3–5). Bryostatins bind to PKC (6), the cellular receptor for phorbol esters, but unlike the phorbol esters, which are tumor promoters, bryostatins lack tumor-promoting properties and under certain conditions can inhibit tumor promotion mediated by phorbol esters (7).

In view of the unique activities of bryostatins, including their potent immunomodulatory effects and antineoplastic properties, we have evaluated the effects of bryostatin 1 against B16 melanoma, a tumor for which immune mechanisms play an important role in its eradication. Our results described here show that bryostatin 1 is effective against B16 melanoma in vivo.

MATERIALS AND METHODS

Animals. Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed under sterile conditions. Mice were 8–10 weeks of age when used for experiments.

Tumor Cell Lines. The B16 murine melanoma cell line was obtained from the Frederick Cancer Research Facility and grown as an adherent monolayer. The NK cell-sensitive Moloney virus-induced YAC-1 lymphoma cell line and REH, a human leukemia cell line, were grown as suspensions (8). Cells were maintained in culture using Eagle’s minimal essential medium, supplemented with 10% heat-inactivated fetal calf serum, nonessential amino acids, vitamins, 1 mM sodium pyruvate, and 1 mM L-glutamine.

Treatment. Bryostatin 1 was isolated from the marine bryozoan Bugula neritina as previously described (1). Stock bryostatin 1 was maintained at −20°C and, when needed, was resuspended in PBS with DMSO (<0.1%) just before use. TPA was purchased from Sigma Chemical Co. (St. Louis, MO), dissolved in DMSO, and diluted in PBS. The final concentration of DMSO was <0.1%. rIL-2 produced in Escherichia coli was kindly supplied by Cetus Corporation (Emeryville, CA). Each vial contained 1.5 × 10^6 units of rIL-2 as a lyophilized powder. Sterile water was used to reconstitute the product.

Therapeutic Experiments. B16 tumor cell suspensions were prepared by trypsinization. The cells were washed 3 times with Hanks’ balanced salt solution. Cell count and viability were determined by trypsin blue dye exclusion. On day 0, mice were given injections of 5 × 10^5 cells/0.2 ml PBS i.v. using the tail vein. This number of tumor cells establishes approximately 100 microscopic melanoma pulmonary nodules by day 3 and macroscopic melanoma pulmonary foci after 12–15 days in untreated mice. Three days after tumor injection, animals were randomized into treatment groups, including bryostatin 1, TPA, and rIL-2. All treatments were given as i.p. injections in 0.5 ml. The dose of rIL-2 administered was 25,000 units every 8 h (9). Control animals received PBS with 0.1% DMSO (diluent) in the same manner as the treated animals. All treatments began on day 3 following tumor injection and continued for 5 days. The mice were monitored daily for morbidity and mortality. On day 14 after tumor inoculation the mice were sacrificed. The melanoma pulmonary metastases appeared as discrete black nodules formed on the surface of the lungs. The lungs were removed and melanoma nodules were counted in a blinded fashion with the aid of dissecting microscope.

Preparation of Murine Splenocytes. C57BL/6 mice were sacrificed and their spleens were aseptically removed, pooled, and passed through sieves of 40 μm and 70 μm screen. The cells were washed 3 times with PBS and resuspended at a concentration of 10^7 cells/ml. An aliquot of 1.0 ml was injected i.p. to each mouse. The cost of publication of this article was 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.

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The abbreviations used are: PKC, protein kinase C; NK, natural killer; LAK, lymphokine-activated killer; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; rIL-2, recombinant interleukin 2; IL-2, interleukin 2; TPA, 12-O-tetradecanoyl-13-phorbol acetate.

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a mesh screen with the blunt end of a 10-ml syringe plunger. The cell pellet was obtained and the RBC were lysed with Tris-NaCl. The cell suspension was then centrifuged over fetal calf serum and washed 3 times in PBS. These effector cells were incubated for various time periods with bryostatin 1 (1 x 10^{-8} M) and/or rIL-2 (1000 units/ml) prior to cytoxicity assays.

Induction of LAK Cells. C57BL/6 murine splenocytes (5 x 10^6 cells/ml) were cultured in 25 ml of medium (as above) in 75-cm^2 flasks in the presence of rIL-2 (1000 IE/ml) (9), with or without bryostatin 1 (1 x 10^{-8} M), for 3 days at 37°C in a 5% CO_2 atmosphere. LAK cell cytotoxicity was measured against B16 melanoma cells using a chromium release assay.

Assay for Cytotoxicity. A 4-h chromium release assay was performed using standard techniques (10). Targets included Yac-1 tumor cells and B16 melanoma cells. Cells (1 x 10^5) labeled with 100 μl of Cr were plated into each well, along with effector cells. Effector consisted of splenocytes from control or tumor-bearing C57BL/6 mice that were incubated for 2 or 72 h with bryostatin 1, rIL-2 (LAK cells), or bryostatin 1 and rIL-2. Effectors were plated in triplicate at three different effector to target cell concentrations, 100:1, 40:1, and 10:1. Maximum and spontaneous Cr release were obtained by adding 0.1 ml of 1 N HCl or 0.1 ml of medium, respectively, to wells with target cells. Percentage of specific Cr release was calculated according to the formula:

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\% \text{ specific Cr release} = \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximal} - \text{cpm spontaneous}} \times 100
\]

Clonogenic Assay. The tumor cell clonogenic assays were performed by culturing 500 to 1000 B16 melanoma cells or REH cells/ml in 0.3% agar, bryostatin 1 (1 x 10^{-8} to 1 x 10^{-4} M), 20% fetal calf serum, McCoy's medium 5A. One ml of cells with agar mixture was plated in 35-mm Petri dishes. The assays were performed in quadruplicate. The plates were scored after 7 days of incubation for B16 melanoma cells and after 18 days for REH cells, using an inverted microscope. A colony was considered an aggregate of 50 or more cells.

Histological Procedures. For histological examination of in vivo tumors and tissues, animals were sacrificed and the relevant tissues were removed aseptically and placed in formalin. The tissues were then sectioned and stained with hematoxylin and eosin and reviewed in consultation with a pathologist.

Statistical Analysis. For the in vivo experiments, the significance of differences in the number of pulmonary metastases between groups was determined by a Wilcoxon rank test. In vitro results were analyzed for statistical significance by analysis of variance and Student's t test.

RESULTS

Effect of Bryostatin 1 on B16 Melanoma Pulmonary Metastases. To establish whether bryostatin 1 could mediate regression of established B16 melanoma pulmonary metastases, C57BL/6 mice were given i.v. injections of tumor cells. Therapy with bryostatin 1 was initiated on day 3 following tumor injection and continued for 5 days. On day 14 animals were sacrificed, lungs were harvested, and pulmonary nodules were enumerated. As shown in Table 1, bryostatin 1 significantly reduced the number of pulmonary nodules, compared to the control animals that received diluent (PBS with 0.1% DMSO) (P < 0.0001). Histological examination of the lungs revealed the presence of tumor cell foci in sections. Both experimental and control lungs exhibited modest mononuclear cell infiltration; however, the degree of cellular infiltration was not different between the two groups. The size of the remaining nodules in bryostatin 1-treated mice was significantly smaller. There was no evidence of necrosis and the residual nodules were pigmented with melanin.

The data in Fig. 1 indicate the inhibitory dose-response effect of bryostatin 1. The maximal reduction in the number of pulmonary nodules was achieved at a bryostatin 1 dose of 100 μg/kg/day (95% reduction). At 50 μg/kg/day, bryostatin 1 resulted in a 76% reduction in nodules, and even at 1 μg/kg/day, the lowest dose evaluated, bryostatin 1 had a significant antimelanoma effect when compared to the control treatment (P < 0.01). Not only was the number of nodules in bryostatin 1-treated animals effectively reduced, but the size of the residual nodules also was smaller in the bryostatin 1-treated animals, when compared to the nodules in the control animals receiving diluent (Fig. 2). These results suggest that an optimal antimelanoma dose of bryostatin 1 is approximately 100 μg/kg/day. Of note, animals treated with doses greater than 50 μg/kg bryostatin 1 developed a transient clinical syndrome consisting of diarrhea, weight loss, and oliguria. These toxicities were not encountered at the lower dose levels. Bryostatin 1 doses greater than 200 μg/kg were uniformly lethal within 24 h of administration. Autopsy studies in these animals failed to reveal a definite cause of death.

Effect of Bryostatin 1 and TPA on Established B16 Melanoma Pulmonary Metastases. Because bryostatin 1 shares many biological effects with the phorbol esters, we were interested in comparing the antimelanoma effects of bryostatin 1 to those of phorbol esters. C57BL/6 mice were inoculated with B16 melanoma cells on day 0. On days 3–7 the mice were treated with either 100 μg/kg/day bryostatin 1, 100 μg/kg/day TPA (a phorbol ester), the combination, or diluent. As shown in Fig. 3, bryostatin 1 significantly reduced the number of pulmonary nodules. In contrast, there was no antimelanoma effect observed following TPA treatment. The combination of both agents administered simultaneously was highly toxic, since all animals...
Fig. 2. Photograph of the lungs from melanoma-bearing animals. C57BL/6 mice were treated as in Fig. 1. Left, control animal receiving diluent injections on days 3–7. Right, animal receiving bryostatin 1 (100 μg/kg/day) on days 3–7.

Fig. 3. Effects of experimental therapies on pulmonary nodules. C57BL/6 mice were given injections of 5 x 10⁴ B16 melanoma tumor cells. On days 3 through 7, mice were treated as follows. Left, bryostatin 1 (BRYO) (100 μg/kg/day), TPA (100 μg/kg/day), or diluent (n = 8 for each treatment group). Right, bryostatin 1 (50 μg/kg/day), rIL-2 (25,000 units every 8 h), bryostatin 1 and rIL-2 (same doses), or diluent (n = 6 for each treatment group). On day 14, animals were sacrificed and pulmonary metastases were counted. Values shown are the mean number of nodules.

Expired within 24 h of the treatment. Autopsy studies did not reveal a definite cause of death.

Effect of Bryostatin 1 and rIL-2 on B16 Melanoma Pulmonary Metastases. Early reports have demonstrated synergy between bryostatin 1 and IL-2 in terms of T-cell activation and cytotoxicity (11). Therefore, we evaluated whether IL-2 could also enhance the antimelanoma effect of bryostatin 1. Animals were given injections of tumor, as described. Tumor-bearing mice were treated with bryostatin 1 (50 μg/kg), bryostatin 1 (50 μg/kg) plus rIL-2 concurrently, rIL-2 alone, or diluent, beginning on day 3, for 5 days as per protocol. Bryostatin 1 was given once a day while the rIL-2 (25,000 units) was given 3 times a day. As shown in Fig. 3, animals treated with bryostatin 1 had an 86% reduction of pulmonary metastases, while rIL-2 therapy alone caused only a 27% reduction. Combination of bryostatin 1 and rIL-2 resulted in a 27% reduction of pulmonary nodules, indicating no enhanced antitumor effect for this combination. In fact, the data suggest that rIL-2 interfered with or antagonized the antimelanoma effect of bryostatin 1, since equivalent doses of bryostatin 1 resulted in a decreased therapeutic effect when combined with rIL-2. Additional experiments were conducted to evaluate synergy between rIL-2 and bryostatin 1. For those experiments, tumor-bearing mice were sequentially treated with bryostatin 1 followed by rIL-2 (i.e., 5 days of rIL-2 followed by 5 days of bryostatin 1 or vise versa). No enhanced antitumor effect for the combination was observed (data not shown).

Effect of Bryostatin 1 on Growth of B16 Melanoma Cells in Vitro. To examine whether the antineoplastic property of bryostatin 1 in whole animals results from a direct antiproliferative effect, a clonogenic assay was performed. B16 melanoma cells and REH cells, a line sensitive to bryostatin 1 in culture, were cultured in 0.3% agar in the presence of increasing concentrations of bryostatin 1 from 1 x 10⁻¹⁰ M to 1 x 10⁻⁶ M. Fig. 4
In Vitro Cytotoxic Activity of Mononuclear Cells against B16 Melanoma Cells. B16 melanoma has been shown previously to be sensitive to cell-mediated immune responses and immune interventions in vivo (12–14). We were, therefore, interested in examining whether the suppression of melanoma pulmonary nodules in vivo by bryostatin 1 could potentially result from enhanced lymphocyte cytotoxicity. In the first set of experiments, we evaluated the capacity of C57BL/6 splenocytes incubated in vitro with bryostatin 1 to lyse melanoma or Yac-1, an NK-sensitive cell line. The data, shown in Fig. 5, indicate that bryostatin 1 did not enhance splenocyte cytotoxicity against B16 melanoma. As expected, Yac-1 target cells were sensitive to lysis by normal murine splenocytes. This lytic activity, however, was inhibited when splenocytes were incubated with bryostatin 1.

Since splenocytes incubated with bryostatin 1 were unable to mediate lysis of B16, we examined whether bryostatin 1 treatment in vivo could generate an antimelanoma effect in vitro. Thus, spleen cells obtained from C57BL/6 mice which had been treated with bryostatin 1 (75 μg/kg) or control diluent daily for 5 days were tested for in vitro antimelanoma cytotoxicity. The percentage of lysis at effector to target ratios of 100:1, 40:1, and 10:1 was 8%, 6%, and 6%, respectively, which indicates that bryostatin 1 treatment in vivo was unable to enhance splenocyte-mediated antitumor activity in vitro. These results were not different whether splenocytes were isolated from tumor-bearing or non-tumor-bearing animals (data not shown).

Finally, we sought to determine the effect of bryostatin 1 on the generation of LAK cell activity in vitro. Splenocytes were cultured in the presence of rIL-2 (1000 units/ml) (positive control) and/or bryostatin 1 (1 × 10^{-8} M) for 3 days, and cytotoxicity against B16 melanoma was assessed. This concentration of bryostatin 1 was utilized because previous studies have shown this to be the optimal concentration for activation of lymphocytes (3). The data are shown in Fig. 5. IL-2, but not bryostatin 1, induced LAK cell activity from murine splenocytes when tested against B16 melanoma targets. Nevertheless, the addition of bryostatin 1 inhibited the ability of rIL-2 to generate LAK cell activity, since splenocytes treated with bryostatin 1 plus rIL-2 displayed no lytic activity against B16 melanoma.

**DISCUSSION**

The results from this study reveal that short term treatment of animals with bryostatin 1 causes a dramatic reduction in the number of established pulmonary melanoma metastases. Dose escalation studies clearly demonstrate an antitumor dose-response effect for bryostatin 1, with 100 μg/kg/day being the most effective dose (Fig. 1). The sensitivity of this tumor in vivo to bryostatin 1 is exquisite, apparent even at very low nontoxic doses of 1 μg/kg/day. This finding suggests that combining bryostatin 1 with other anticancer therapies may be easily accomplished without significant toxicity.

The primary question raised by our results is the mechanism of bryostatin’s therapeutic effect. Our results demonstrate that there is significant direct inhibitory effect on the growth of B16 melanoma cells in culture (Fig. 4), but only at the highest concentration tested. This is consistent with previously published data, in which direct antiproliferative effects of bryostatin 1 were shown against a number of cancer cell lines (1, 15). The concentration of bryostatin 1 necessary to achieve in vitro antiproliferative effects against B16 melanoma, however, is substantially higher than that required to inhibit other sensitive cell lines (1). This suggests that direct antiproliferative activity of bryostatin 1 in vivo may not be entirely responsible for the antineoplastic effect observed. The ability to monitor serum and intracellular levels of bryostatin 1 would help to clarify the differences in the concentrations of bryostatin 1 necessary for the in vivo and in vitro antitumor effect, but many technical limitations in performing these assays have not yet been resolved.

Previous reports have confirmed the important role of immune mechanisms in the inhibition of B16 melanoma tumor growth. Biological response-modifying agents such as interferon and rIL-2, as well as cell-mediated cytotoxicity by LAK cells, have all been reported to inhibit B16 melanoma in vivo (12–14). Recent reports have confirmed that bryostatin 1 has potent immunoenhancing properties (11). Data from our laboratory demonstrate that bryostatin 1 causes activation of T-cells and can induce the expression of functional IL-2 receptors on human CD4+ and CD8+ lymphocytes (3). Other investigators have reported that bryostatin 1 can stimulate resting T-cells to proliferate and differentiate into cytotoxic T-lymphocytes (11).

Based upon these data, we investigated whether such immune mechanisms might be involved in the observed antitumor effect of bryostatin 1. In a series of experiments, we tested whether bryostatin 1 could enhance cell-mediated cytotoxicity against B16 melanoma. However, our experiments indicate that, at least in the in vitro setting, bryostatin 1 does not stimulate an antitumor cell-mediated immune response. Thus, C57BL/6 splenocytes incubated with bryostatin 1 for 2

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**Fig. 5.** Cell-mediated cytotoxicity against B16 melanoma of splenocyte effector cells incubated for 2 or 72 h with bryostatin 1 (1 × 10^{-8} M) (Bryo), rIL-2, or diluent. Spleen cells were obtained from 8–10-week-old C57BL/6 mice, and single-cell suspensions were treated as indicated. These cells were then washed 3 times and tested for cytotoxic activity against "Cr-labeled B16 melanoma, as described in “Materials and Methods.”
to 72 h fail to mediate cytotoxicity against B16 melanoma (Fig. 5). In addition, bryostatin 1 was also found to inhibit NK cell cytotoxicity (Fig. 6), because normal splenocytes treated in vitro with bryostatin 1 had lower NK activity than untreated control cells. As has been previously reported, splenocytes which are cultured in the presence of rIL-2 cause proliferation and generation of LAK cells that can lyse a variety of tumor cells, including B16 melanoma (9). This was confirmed in our studies, as indicated in Fig. 5. As the data in Fig. 5 indicate, however, bryostatin 1 interfered with the generation of LAK cells. Splenocytes incubated with IL-2 showed pronounced lytic activity against B-16, but this was strongly inhibited by the inclusion of bryostatin 1. Thus, bryostatin 1 has dual effects on immune function, stimulating T-cell proliferation and cytotoxicity while inhibiting LAK and NK cell activity. This is consistent with the data recently reported by Nishimura et al. (16), indicating that agents that activate PKC can interfere with the generation of LAK cells by IL-2 but enhance IL-2-induced cell proliferation and IL-2 receptor expression on lymphocytes. As suggested by these studies, it is possible that bryostatin 1 treatment causes PKC desensitization or down-regulation of the PKC receptor on lymphocytes (17). This may impede any PKC-dependent signal transduction necessary for bryostatin 1 effects.

We also evaluated whether rIL-2 and bryostatin 1 in combination could enhance the antimalanoma effect of bryostatin 1. Treatment of mice with rIL-2 alone caused only a marginal reduction in melanoma pulmonary metastases. Moreover, combining rIL-2 and bryostatin 1 was apparently antagonistic, because animals receiving this combined treatment had a greater number of pulmonary nodules than mice treated with equivalent doses of bryostatin 1 alone (Fig. 3). This antagonistic effect cannot be explained by the available data, although a similar inhibitory interaction has been previously reported by Trenn et al. (11), who found that low concentrations of bryostatin 1 enhanced IL-2 cytotoxicity but high concentrations of bryostatin 1 either had no effect or inhibited IL-2 cytotoxicity.

Previous studies have reported that bryostatins share some biological properties with phorbol esters. Both bryostatin 1 and TPA are equipotent activators of PKC in mediating phosphorylation of cell surface protein receptors, including the transferrin receptor, and in activating T-cells and neutrophils (3, 6, 11, 18). These findings suggest that the biological effects of bryostatin 1 are similar to those of phorbol esters and may be mediated through activation of PKC, although marked differences in the biological effects of these two agents have been reported (19, 20). Bryostatin 1 blocks phorbol ester-induced differentiation of certain cell lines (21), possess antineoplastic effects (22), and can mimic certain stimulating effects of hematopoietic growth factors (5) and, whereas phorbol esters are potent tumor promoters, bryostatins lack both first and second stage tumor-promoting activity (7). Both types of agents can inhibit cell growth, but the growth-inhibitory effects of TPA and other phorbol esters against some cell lines appears to be associated with their activation of PKC and induction of terminal differentiation (23). The mechanism whereby bryostatin 1 exerts its antiproliferative activity remains unknown.

Given these effects, we thought it necessary to compare bryostatin 1 with TPA in terms of an antimalanoma effect. Our data reveal that treatment of animals with bryostatin 1 for 5 days consistently reduced the number of established pulmonary metastases, whereas treatment with TPA at equivalent doses had no effect on tumor growth (Fig. 3). The data from these experiments further support the divergent effects of TPA and bryostatin 1 on tumor cell growth. Whether the antimalanoma activity of bryostatin 1 is independent of PKC activation or perhaps involves the differential activation of PKC isozymes (24) is unknown at present but remains an intriguing topic for further studies.

In conclusion, our results demonstrate that bryostatin 1 can have direct antiproliferative effects in vitro against B16 melanoma. Since bryostatin 1 can also inhibit B16 melanoma tumor growth in vivo, it is likely that this is, at least in part, the result of a direct effect. Furthermore, in our experimental setting we could not find any evidence that bryostatin 1 can stimulate nonspecific cell-mediated cytotoxicity against B16 melanoma in vitro, despite the potent immunoenhancing properties of bryostatin 1 (3, 11). Although bryostatins share many biological properties with phorbol esters, our data demonstrate certain divergent biological effects of bryostatin 1 and phorbol esters. Whether the significant therapeutic effects of bryostatin 1 result solely from a direct antiproliferative effect is unknown. It is also possible that indirect immunomodulatory effects (i.e., release of tumor necrosis factor or other cytokines), cytotoxic T-lymphocytes, or other factors may be involved in the antimalanoma effect observed in vivo. Further investigation into the mechanism of action of these new agents will likely lead to improvements in the treatment of human cancers such as melanoma.

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