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Comparison of the antitumor activity of bryostatins 1, 5, and 8

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Abstract Bryostatin 1, a macrocyclic natural lactone isolated from a marine Bryozoan, has undergone phase I testing in humans. Side effects of treatment have included muscle pain and joint aches, a transient decrease in platelets, and the release of tumor necrosis factor alpha (TNFα) and IL-6 into the blood stream. In animals, anticancer activity has been demonstrated against murine leukemias, lymphomas, melanomas, and sarcomas. The mechanism of action of this compound depends in part on its ability to activate protein kinase C. To determine the biologic activity and toxicity of other members of the family of bryostatin compounds, we studied the ability of bryostatins 5 and 8 to inhibit the growth of murine melanoma K1735-M2. Bryostatins 1, 5, and 8 induced equivalent inhibition of melanoma growth, but bryostatins 5 and 8 induced less weight loss than bryostatin 1 ($P < 0.001$). Neither the injection of an antimurine TNFα antibody nor an adenovirus, which produces a mutated TNF receptor inhibiting TNFα activity, into mice had any effect on either bryostatin-induced weight loss or melanoma tumor growth inhibition. Using a novel competition assay, the levels of bryostatin in the plasma were measured. The approximate half-life ($t_{1/2}$) of bryostatin was 8.62 min, the clearance (Cl) 3.53 ml/min and the AUC 322.20 nmol/l min. A similar result was obtained with each bryostatin analog. These results suggest that human testing of additional bryostatin analogs may yield compounds with similar antitumor activity but decreased side effects. A novel assay to measure the level of all bryostatins in the plasma of patients undergoing treatment is described.

Keywords Bryostatin analogs · Protein kinase C
Tumor necrosis factor · Bryostatin blood levels

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Introduction

Recently phase I anticancer trials in humans of the novel anticancer agent bryostatin 1 have been carried out [1, 2]. Bryostatin 1 is a naturally occurring macrocyclic lactone derived from a marine Bryozoan, *Bugula neritina* [3]. In animals it has antitumor activity against murine tumors, including B16 melanoma [4], M 5076 reticulum cell sarcoma [5], L10A B-cell lymphoma [6], and P388 lymphocytic leukemia [5]. Bryostatin 1 inhibits clonogenic growth of K562 cells (a myeloid leukemia cell line), REH cells (a pre-B-lymphoblastic cell line), and fresh acute nonlymphocytic leukemia cells, but it shows only marginal activity against clonogenic CEM cells (a T-lymphoblastic cell line) [7]. The mechanism of action of bryostatin 1 is related to its ability to activate protein kinase C (PKC) in tissue culture cells, causing the translocation of this enzyme to the membrane [8] and the phosphorylation of specific protein substrates [9]. As with other PKC activators, prolonged incubation of bryostatin 1 leads to the eventual degradation of PKC. Bryostatin 1 does not affect all PKC isoforms in an equivalent fashion [10]. Although it induces translocation of α, δ and ε isoforms, the α isoform is degraded much more rapidly [11].

In phase I trials the major side effect of bryostatin 1 has been found to be muscle aches and joint pains [1, 2]. These changes are not associated with any abnormality in EMG or muscle enzymes and are the dose-limiting toxicity. At the highest doses infused (50 $\mu g/m^2$), bryostatin 1 induces a significant increase in tumor necrosis factor α (TNFα) and interleukin-6 [1, 2] in the plasma, and a transient decrease in the white cell count and platelets. Similarly, in tissue culture bryostatin 1 induces an increase in TNFα levels, which may be responsible for the inhibition of growth of chronic myelomonocytic leukemia cells [2]. Thus, both the antitumor activity and the side effects of bryostatin 1 could be mediated by the release of specific hormones secondary to the stimulation of protein kinase C, for example TNFα.

The bryostatins are a large family of compounds with varying side chains off the macrocyclic lactone ring [5]. Although all bryostatins activate PKC, these close structural relatives have varied biologic activity. Bryostatins 2 and 3 stimulate a significantly greater increase in arachidonic acid release from murine C3H $10^{1/2}$ cells than either bryostatin 1 or 4 [12]. In contrast, epidermal growth factor binding to its receptor is inhibited to a similar degree by bryostatins 1, 2, 3, and 4 [12]. The addition of bryostatin 1 to SH-SY5Y neuroblastoma cells inhibits their DNA synthesis at a tenfold lower concentration than bryostatin 2 [13]. Similarly, bryostatin 1 is tenfold more active than bryostatin 2 in inhibiting the growth of lung carcinoma cells [14]. Although bryostatin 2 is capable of inhibiting the growth of GH_4 cells, bryostatin 1 has no effect [15]. Bryostatins 3 and 8 have identical effects on cell growth to bryostatin 1 [15]. Bryostatin 3 also enhances cell-substratum adhesion, whereas, both bryostatin 8 and 1 have no effect on this property [15]. These results suggest that closely related bryostatins could have different antitumor or other effects when examined in animal tumor models.

Because other bryostatin analogs have the potential for important clinical use but may have a different spectrum of toxicity from bryostatin 1, which is currently undergoing clinical testing, we examined the antitumor activity of bryostatins 5 and 8 (Fig. 1). To aid in this study we developed a novel bioassay to measure the level of bryostatins in plasma, and studied whether the release of TNFα mediates either the antitumor activity or weightloss induced by the bryostatins.

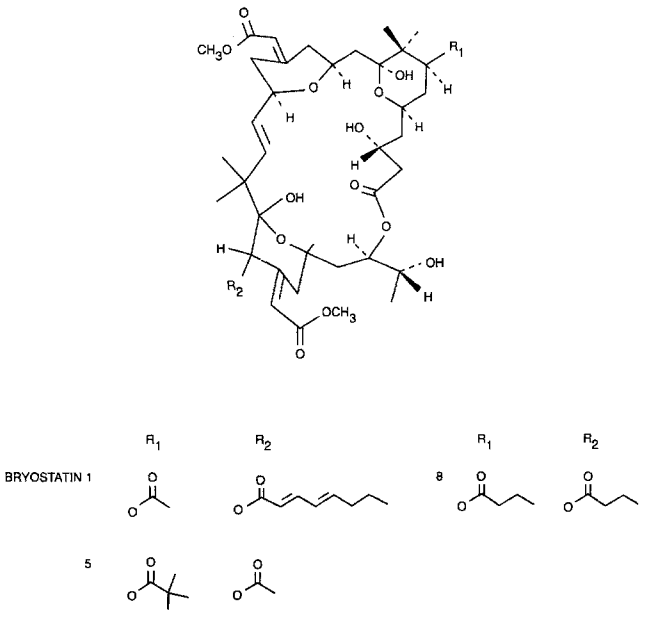

Fig. 1 Structure of bryostatin analogs. R_1 and R_2 denote the side chains

Materials and Methods

Chemicals and reagents

Bryostatins 1,5, and 8 were dissolved at a stock concentration of 1 m*M* in DMSO and stored at -20°C. Prior to injection of each bryostatin, they were dissolved in 10% DMSO/PBS. RPMI was obtained from Gibco Laboratories (Grand Island, N.Y.); fetal calf and bovine serum from HyClone Laboratories (Logan, Utah); and ^{3}H-phorbol dibutyrate (^{3}H-PDBu) from Amersham (Arlington Heights, Il). All other reagents were purchased from Sigma Chemical (St. Louis, Mo.).

Animal tumors

The melanoma tumor cell line K1735 M2 clone 10 was provided by Dr. Isaiah J. Fidler (Department of Cell Biology, M.D. Anderson Cancer Center, Houston, Tx.) and maintained in RPMI containing 5% fetal bovine serum, L-glutamine, nonessential amino acids, sodium pyruvate, and antibiotics. Prior to injection, cells were trypsinized and washed three times with PBS. The tumors were then established by the injection of 1×10^6 cells/0.2 ml PBS into the tail vein of C3H/HeN mice (Charles River Laboratories, Raleigh, N.C.). Three days after tumor injection, mice were begun on daily intraperitoneal (i.p.) injections of different bryostatins (1 μg per mouse for each bryostatin) in 0.2 ml 10% DMSO/PBS for a total of 10 days. On day 14 after the injection of tumor cells, the animals were weighed, the lungs removed, the left lung weighed, and a section of the right lung examined pathologically.

Competition assay to measure bryostatin levels in plasma

Rat membrane preparations as a source of protein kinase C (PKC) were prepared as described previously [16]. Two rat brains were placed in 10 ml ice-cold homogenization buffer (10 m*M* Tris-HCl, pH 7.5). The brains were dounce homogenized with 25 strokes, and the homogenate was spun at 17,000 rpm for 10 min at 4°C. The pellet was washed three times in 10 ml homogenization buffer. This step was repeated two more times. The final pellet was resuspended in 10 ml homogenization buffer and frozen in 1 ml aliquots at − 20°C for future use. To assay bryostatin levels, 1 μl ^{3}H-PDBu in 700 μl assay buffer (50 m*M* Tris-HCl, pH 7.5, 2 mg/ml bovine serum albumin) was used. The frozen rat brain stock was diluted 1:10 in assay buffer, and 100 μl was used per assay (approximately 25 μg wet

wt protein). Varying concentrations of bryostatin(s) were added as competitors in 200 µl assay buffer to give a final reaction volume of 1 ml. After incubation for 60 min at 30°C, the reaction was stopped by filtering over a GFC filter. The filters were washed with three 10-ml aliquots of 5% trichloroacetic acid (TCA) and then counted.

To measure the level of the bryostatins in plasma, they were injected intravenously (in 0.2 ml 10%DMSO/PBS) into the tail vein of a C3H/HeN mouse. At the times indicated, the mice were bled from the venous plexus behind the eye. The blood was collected into tubes containing citrate as an anticoagulant. The measurement of bryostatin levels was done as described above in triplicate except that 100 µl plasma plus 100 µl assay buffer were added instead of stock bryostatin.

TNF levels

Plasma TNF was measured using the WEHI 164 subclone 13 cytotoxicity assay [17]. Briefly, 5×10^4 cells growing in RPMI-1640 containing 10% fetal calf serum, 1 m*M* L-glutamine and 0.5 µg/ml actinomycin D were added to serially diluted samples in microtiter plates and incubated for 20 h at 37°C in an atmosphere containing 5% CO_2. Cytotoxicity was assessed by measuring the death of L929 cells using the conversion of MTT-tetrazolium (3,[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide) to [CEZ] formazan at 550 nm [18]. TNF concentrations (pg/ml) were calculated using standard recombinant muTNFα (Genentech, South San Francisco, Calif.), as goat anti-muTNFα IgG reduces plasma TNF activity to undetectable levels.

Adenovirus propagation

Human kidney 293 cells 40 15-cm plates were infected with adenovirus at 10^9 plaque–forming units (pfu). After approximately 48 h the cells were harvested and then freeze-thawed five times to release the virus. The virus was purified over cesium chloride and then twice over a Sepharose CL-6B spin column. To quantitate the viral yield a plaque assay was done on the 293 cells. After 30 min of infection the virus was removed from the plate and the monolayer overlain with 2 ml agar-containing medium. The plaques were counted after 7 days and the titer determined. Each mouse was injected with 10^9 pfu.

Pharmokinetic analysis

The pharmokinetic parameters of bryostatin 1 were estimated by model-independent methods using the SIPHAR program [19]. The AUC was calculated by the trapezoidal rule with extrapolation to time infinity by the use of the terminal disposition slope (k) generated by weighted nonlinear least-squares regression, [20] with the weighted factor set as the reciprocal of the calculated concentration squared. Elimination $t_{1/2}$ values were calculated from $0.693/k$. The total plasma clearance (Cl) of bryostatin 1 was calculated by dividing the dose by the AUC.

Results

In vivo antitumor activity of bryostatins 1, 5, and 8

Because of the differing in vitro biologic effects of the various bryostatin analogs we designed experiments to examine the ability of bryostatin 5 or 8 to inhibit the growth of murine melanoma in vivo. These analogs were chosen because at high doses they inhibit the growth of these melanoma cells in tissue culture (data not shown). As described in the Methods, mice were injected with melanoma cells and 3 days later a 10-day course of daily i.p. bryostatin was given. Because intravenously injected melanoma cells lodge in the lungs, lung weights were used as a measure of chemotherapeutic efficacy. As demonstrated by the lung weights, both bryostatin 5 and 8 inhibited the growth of murine melanoma to the same degree as bryostatin 1 (Table 1). The weight of lungs after treatment with bryostatin 5 and 8 were not significantly different from the weight of lungs of naive controls which had not been injected with tumor. However, microscopic examination of lung sections from animals injected with tumor and the bryostatins demonstrated small nests of tumor cells remaining after treatment (data not shown). Therefore, 10 days of daily bryostatin injection was not sufficient to completely eradicate the large number of melanoma cells given to these mice.

As reported previously [21], the injection of bryostatin 1 caused significant weight loss in comparison with control tumor-bearing mice when injected i.p. over 10 days ($P < 0.0001$ when compared with uninjected controls). As shown in Table 1, the injection of equivalent amounts of bryostatins 5 and 8 caused less weight loss than bryostatin 1 ($P < 0.001$), although bryostatins 5 and 8 still induced weight loss ($P < 0.01$) when compared with the weight of the control mice injected with tumor. These mice were able to retain

Table 1 The effect of bryostatins 1, 5, and 8 on the growth of murine melanoma in vivo. Values are means ± SE

	Uninjected[a] ($n = 8$)	Control[b] ($n = 16$)	Bryostatin 1 Treatment[c] ($n = 19$)	Bryostatin 8 Treatment[c] ($n = 10$)	Bryostatin 5 Treatment[c] ($n = 8$)
Animal weights (g)	22.26 ± 0.35	21.15 ± 0.52	15.10 ± 0.58**	19.49 ± 0.32*	17.59 ± 0.44*
Lung weights (g)	0.07 ± 0.006	0.22 ± 0.031	0.07 ± 0.006**	0.102 ± 0.008**	0.07 ± 0.013**

[a]Mice received no tumor cells or bryostatin, were weighed and sacrificed
[b]Mice were injected with 10^6 melanoma cells and weighed on day 14
[c]Mice were injected with 10^6 melanoma cells and then with 1 µg bryostatin i.p. from day 3 to 13 and sacrificed on day 14
*$P < 0.001$ vs bryostatin 1 **$P < 0.0001$ vs control

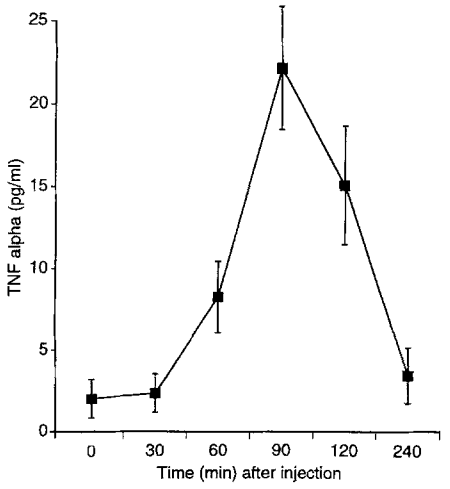

Fig. 2 TNFα levels in the plasma of mice after bryostatin 1 injection. At the times indicated three mice were bled and TNFα levels determined in triplicate in each sample as described in Materials and methods. The standard deviation and the mean of these values are shown

83% and 92% of their body weight, respectively, as compared with 71% for the bryostatin 1-treated animals. Injections of bryostatins 1, 5, and 8 did not cause any mortality, and stopping the injections led to an increase in body weight (data not shown).

The effect of inhibition of TNFα activity on bryostatin, induced weight loss and tumor regression

To evaluate whether bryostatin 1 stimulates the release of TNFα in mice, bryostatin 1 was injected intravenously, and blood was drawn at varying times after injection. Although the elevation in TNFα levels induced by bryostatin 1 was small, an elevated level was easily measured at 90 and 120 min after injection (Fig. 2). To evaluate whether differences in the levels of TNFα induced by bryostatins 1, 5, and 8 could account for some of the differences in biologic behavior of these compounds, each of these analogs was injected into mice, and blood drawn at 120 min after injection was analyzed. However, no significant difference was found between the levels of TNFα induced by either bryostatin 5 or bryostatin 8 when compared with that induced by bryostatin 1 (data not shown).

The ability of neutralizing TNFα antibodies to inhibit the weight loss or tumor killing induced by bryostatin 1 was evaluated. Animals were injected with tumor cells, and 3 days later they were given bryostatin 1 i.p. for 10 days. This antibody was detected in the plasma 24 h after the injection, suggesting that it had been well absorbed. Five animals also received neutralizing TNFα antibodies i.p. daily for 10 days 60–90 min prior to bryostatin 1. Using an immunoassay, the TNF antibodies could be detected in the plasma 24 h after i.p. injection. At the end of the experiment, there was no significant difference in the weight loss or lung weights between the group of animals receiving bryostatin 1 alone or bryostatin 1 plus the neutralizing antibody to TNFα (Table 2)

The second approach to evaluating the role of TNFα in bryostatin-mediated effects was to use a replication-incompetent adenovirus into which a TNF inhibitor gene had been cloned [22]. This gene encodes a chimeric protein capable of binding and neutralizing TNF and lymphotoxin . Previous work [22] has demonstrated that this virus renders animals susceptible to *Listeria monocytogenes* . The level of the virus is maintained for at least 4 weeks. Mice were injected with 10^9 pfu of this virus, followed the next day by 10^6 1735 M2 melanoma cells. Mice were injected 3 days later with bryostatin 1 i.p. Control groups either did not receive bryostatin 1 or did not receive the adenovirus. Inhibition of TNFα activity by infection with the adenovirus did not affect the bryostatin 1-induced weight loss ($P < 0.0001$ when virally infected plus bryostatin 1-treated mice were compared with control mice) or the bryostatin 1-mediated inhibition of tumor growth ($P < 0.003$ when compared with control mice; Table 3). Thus, these three experiments suggest that both the antitumor activity and the sizeable weight loss induced by bryostatin 1 are not mediated by TNFα.

Table 2 The effect of injection of a TNFα antibody on bryostatin 1-induced weight loss and tumor regression. Values are means ± SE

	Control[a] ($n = 10$)	Bryostatin 1 Treatment[b] ($n = 5$)	Bryostatin 1 plus TNFα Ab[c] ($n = 5$)	Bryostatin 1 plus control plasma[d] ($n = 5$)
Animal weights (g)	20.27 ± 0.38	17.61 ± 0.63*	17.66 ± 0.38*	18.38 ± 0.70*
Lung weights (g)	0.357 ± 0.053	0.114 ± 0.020**	0.112 ± 0.021**	0.154 ± 0.020**

[a]Mice were injected with 10^6 melanoma cells, received no treatment, and were sacrificed on day 14
[b]Mice were injected with melanoma cells plus 1 μg bryostatin 1 i.p. from day 3 to 13
[c]Mice were treated with 1 mg TNFα antibody 60 min prior to bryostatin 1 injection
[d]Mice were treated with 1 mg normal rabbit plasma 60 min prior to bryostatin 1 injection
*$P < 0.0001$ vs control

Table 3 The effect of TNFα blockade by adenovirus-mediated expression of a truncated TNF receptor on bryostatin 1-induced weight loss and tumor regression. Values are means ± SE

	Control mice injected with adenovirus[a] ($n = 6$)	Bryostatin 1 treatment only[b] ($n = 7$)	Bryostatin 1 treatment plus adenovirus ($n = 9$)
Animal weight (g)	21.01 ± 0.767	17.04 ± 0.356**	17.26 ± 0.452**
Lung weight (g)	0.263 ± 0.057	0.108 ± 0.029*	0.098 ± 0.021*

[a]Mice were injected with 10^9 pfu adenovirus followed by 10^6 melanoma cells and sacrificed on day 14
[b]Mice were injected with 10^6 melanoma cells and treated with 1 μg bryostatin 1 i.p. from day 3 to 13 and sacrificed on day 14
[c]Mice were injected with 10^9 pfu adenovirus followed by 10^6 melanoma cells and then with 1 μg bryostatin 1 i.p. from day 3 t 13 and were sacrificed on day 14
*$P < 0.003$, **$P < 0.001$ vs control

Half-life of bryostatins 1, 5, and 8 in murine plasma.

Differences in the biologic activity of bryostatins 1, 5, and 8 may be secondary to differences in the rate of loss of these analogs from the plasma. Previously, we have examined the plasma half-life of bryostatin 1 using a bioassay in which bryostatin 1 in the plasma of injected mice was used to activate human neutrophils [23]. However, this assay is cumbersome and somewhat inexact. We therefore developed an assay which was more sensitive and easier to perform. We took advantage of the technique first utilized to measure bryostatin 1 levels during isolation of this compound from the marine bryozoans [24]. The assay makes use of the ability of bryostatins to compete with phorbol esters for binding to PKC [16]. In this assay, rat brain membranes are used as a source of PKC, and ^{3}H-PDBu is added along with varying amounts of non-radioactive bryostatin . The addition of 100 n*M* bryostatin 1 inhibited the binding of the ^{3}H-PDBu to PKC (Fig. 3) The assay allowed an estimation of levels between 4 and 100 n*M*. No difference was found in the ability of equimolar concentrations of bryostatins 1, 2, 3, 5, 7, and 8 to displace ^{3}H-PDBu from PKC (data not shown).

This assay was then used to measure the half-life of bryostatins in the plasma. Bryostatins 1, 5, and 8 were injected intravenously into the tail vein of different mice. At specific times blood was drawn from the venous plexus behind the eye. To measure bryostatin levels, plasma was added to the reaction mixture, and the level of competition measured. After approximately 30 min the amount of bryostatin 1 in the blood was almost undectectable (Fig. 4). A standard curve was prepared in the presence of 100 μl mouse plasma to help calculate the molarity of bryostatin 1 in the blood. Using purified bryostatins, the variability in this assay was very small (Fig. 3). However, when plasma levels were tested, an increased variability in these measurements was seen. This may be due in part to the observation that plasma decreased the baseline ^{3}H-PDBu by 10%, or to slight differences in injection volume or time of bleeding.

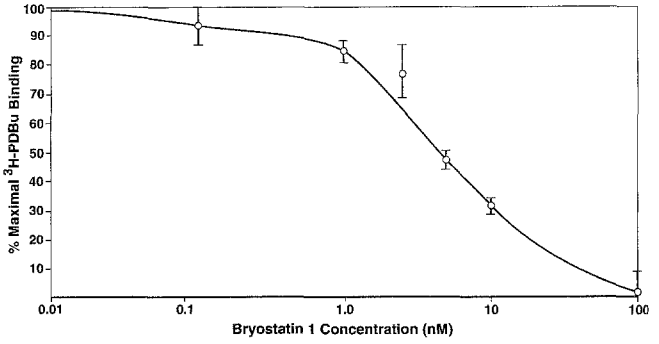

Fig. 3 Competitive binding assay for bryostatin. Various concentrations of bryostatin 1 were added to a mixture of brain membranes and ^{3}H-PDBu and the binding assay was carried out as described in Materials and methods. The counts per minute (c.p.m.) bound to the filters in the presence of bryostatin 1 were divided by the c.p.m. bound in the absence bryostatin and the results expressed as the percentage of maximal binding. The standard error and the means of triplicate experiments are shown

Pharmokinetic parameters were similar for each of the three bryostatin analogs. The apparent plasma elimination times $t_{1/2}$ for bryostatins 1, 5 and 8 were 8.62, 9.39 and 10.67, respectively. The AUCs for each of the bryostatins were as follows: bryostatin 1, 322.20 nmol/l min, bryostatin 5, 399.17 nmol/l min; and bryostatin 8, 381.83 nmol/l min. The total plasma clearances (Cl) for each analog were also similar: bryostatin 1, 3.53 ml/min; bryostatin 5, 3.17 ml/min; and bryostatin 8, 3.07 ml/min.

Discussion

Recent human trials of bryostatin 1 have demonstrated that the side effects of bryostatin 1, muscle pain and joint aches, limit the escalation of bryostatin 1 dosing beyond 50 μg/m^2 [1, 2]. Because other bryostatin analogs might have similar antitumor activity but fewer side effects, we felt that it would be important to test the antitumor activity of bryostatins 5 and 8. The activities of these two analogs were tested in a murine melanoma animal model. In other laboratories bryostatin 1

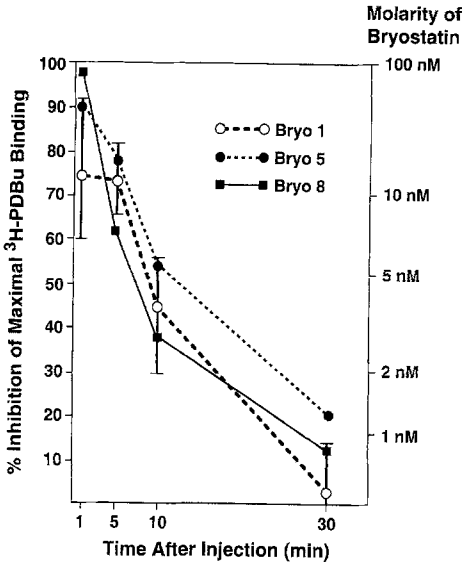

Fig. 4 Bryostatin levels in the plasma of mice. Three groups each of 15 mice were injected in the tail vein with 1 μg of bryostatin 1, 5, and 8, respectively. At the times indicated three mice were bled and the bryostatin levels assayed in triplicate as described in Materials and methods by adding 100 μl plasma to a mixture of brain membranes and ^{3}H-PDBu. The percentage inhibition of maximal ^{3}H-PDBu binding was calculated by subtracting c.p.m. bound in the presence of bryostatin from the value obtained with control plasma. The result was then divided by the c.p.m. bound in the presence of control plasma. For the sake of clarity the standard errors of the values for bryostatin 1 only are shown; the levels of bryostatins 5 and 8 overlap with those of bryostatin 1 at each time point. The molarity of bryostatin 1 derived from a standard curve (see Fig. 3) produced in the presence of 100 μl mouse plasma is shown on the right side of the figure

response has been evaluated done by measuring either the number of melanoma metastases on the surface of the lung [4] or the survival of bryostatin 1-treated and untreated groups [6]. In these studies animals were injected with a 20-fold higher number of melanoma cells so that lung weights reflected tumor growth. Our experiments demonstrate that the lung weights of mice receiving bryostatins 1, 5, and 8 were identical to those of control mice that had not received tumor or bryostatin injections. In comparison, the tumor-bearing controls not treated with the bryostatins had dramatically increased lung weights. Pathologic examination showed that the lungs of the untreated animals were almost completely replaced by melanoma, whereas the lungs of animals treated with bryostatins 1, 5, and 8 showed minimal tumor infiltration. Animals treated with bryostatins 5 and 8, although weighing less than control untreated mice, had significantly less weight loss than those treated with bryostatin 1. Although it is unknown what the major side effect of bryostatins 5 and 8 will be in humans, this result suggests that these two bryostatins might have fewer or different side effects, for example decreased weight loss, while maintaining the same antitumor activity.

Because bryostatin 1 stimulates the release of TNFα in both fresh human leukemic cells in vitro [25] and in vivo [2], we examined the release of this hormone in mice after injection with bryostatin 1. Although the time of maximal release of TNFα in human serum is at 24 h, in mice we measured peak levels at 90 min, which decreased by 4 h. The difference in the time course between mice and humans might result from the prolonged infusion (1 h) of bryostatin 1 in humans versus the rapid i.v. injection in mice. The maximal increase in the plasma TNFα levels (25 pg/ml) in mice is lower than that measured in humans (60 pg/ml). Since lower doses of bryostatin 1 stimulate less TNFα release [2], the difference in the induced level might reflect the differences in the dose used in humans versus the mice. The levels of TNFα released by bryostatins 1, 5, and 8, although low, were not significantly different, suggesting that differences in TNFα release do not account for the difference in side effects induced by these analogs.

Experiments from a number of laboratories have demonstrated that the injection of TNFα prolongs the life of mice carrying the B16 melanoma [26, 27]. In culture, TNFα added with actinomycin D inhibits the growth of the clone of melanoma cells used in these experiments (data not shown). Because bryostatin 1 induces TNFα, experiments were performed to evaluate whether inhibition of TNFα activity blocks the antitumor activity and the weight loss induced by this agent. Antibodies to TNFα have been shown to block the biologic activity of this hormone [28, 29] in a number of different cell systems. However, the injection of TNFα-neutralizing antibody i.p. 60 min prior to bryostatin 1 did not block either the bryostatin 1-induced weight loss or tumor kill.

Another mechanism by which the activity of TNFα can be blocked is by using a truncated receptor protein which binds hormone and prevents it from interacting with cells [30]. Prolonged blockage of TNF activity in mice has been accomplished by making a fusion between the extracellular domain of the human 55-kDa TNF receptor and the murine IgG heavy chain, and expressing this fusion in an adenoviral vector driven by a CMV promoter [22]. Injection of 10^9 pfu of this virus into mice gives a 30-day neutralization of TNFα activity [25]. We also injected 10^9 pfu into mice carrying 1735M2 melanoma. These mice were either treated or not treated with bryostatin 1. Our results demonstrate that blockade of TNFα activity using this virus had no effect on bryostatin 1-induced weight loss or tumor kill, again suggesting that the antitumor effects of bryostatin 1 are not mediated by TNFα. These experiments demonstrate that, while the bryostatins induce the release of TNFα both in vitro and in vivo, inhibition of this activity is not sufficient to block either tumor killing or the side effects of these antitumor compounds.

Using a bioassay based on stimulating the oxidative burst of neutrophils with plasma from mice injected

with bryostatin 1, we have previously shown that 90% of bryostatin 1 activity is removed from the plasma in 2.5 min. Unfortunately, this assay was relatively insensitive and cumbersome. Because human studies of this compound are being carried out it is important to develop a bioassay to measure bryostatin pharmacology in the plasma. To compare the half-life of the three bryostatins examined, we adapted a competitive binding assay first used to purify bryostatin 1 from the marine Bryozoan [16] based on the ability of bryostatin 1 to compete with ^{3}H-PDBu for binding to rat brain membranes. The assay was used to measure bryostatin 1 in plasma samples, and it demonstrated a bryostatin 1 $t_{1/2}$ of approximately 9 min, a Cl of 3.53 ml/min and an AUC of 322.20 nmol/l min with little difference between bryostatins 1, 5, and 8. Because of the ease of performing this assay and the availability of the reagents, It may prove useful in determining the clinical pharmacology of bryostatin in humans. However, it is not quantitative, but gives an accurate estimate of the levels of bryostatin 1 in the plasma. Further improvements in the assay could come from replacing the rat brain membranes with cloned PKC possibly immobilized on beads, competing against ^{3}H-bryostatins rather than ^{3}H-PDBU , and adding an organic extraction step to remove bryostatin from plasma which seems to be inhibitory in this assay.

This work suggests that human testing of additional bryostatin analogs, for example bryostatins 5 and 8, may yield analogs with equivalent anticancer activity and decreased toxicity. A simple bioassay for estimating the level of the bryostatins in plasma is presented.

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