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Biological activity of 26-succinylbryostatin 1

Gary S. Bignami [a], Fred Wagner [b], Paul G. Grothaus [a], Pradip Rustagi [b], Dana E. Davis [a], Andrew S. Kraft [b,*]

[a] *Hawaii Biotechnology Group, Inc., Aiea, HI 96701, USA*
[b] *Division of Hematology / Oncology, University of Alabama at Birmingham, Birmingham, AL 35294, USA*

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Abstract

Bryostatin 1, a macrocyclic lactone, has undergone phase I trials as an anticancer agent. Because of the lipid solubility of this compound it must be delivered either in ethanol or in a PET formulation. During the trial, these vehicles caused a large number of treatment-related side effects. We have synthesized the triethanolamine salt of 26-succinylbryostatin 1 and find that this compound is approx. 100-fold more water soluble than bryostatin 1. Because of the potential for clinical use, we have evaluated the biologic activity of this compound. We find that in a concentration-dependent manner 26-succinylbryostatin 1 is capable of activating protein kinase C (PKC) in vitro and displacing [^{3}H]PDBu from PKC. However, at all concentrations tested the activity was less than the parent compound bryostatin 1. Addition of bryostatin 1 but not 26-succinylbryostatin 1 to U937 leukemic cells in culture stimulated a drop in cytosolic PKC, secondary to translocation of PKC to the membrane. Although 26-succinylbryostatin 1 did not stimulate a drop in the cytosolic levels of PKC, addition to U937 cells activated transcription from an AP-1 enhancer construct and c-Jun protein phosphorylation in a similar fashion to bryostatin 1 and differentiation of U937 cells. Unlike bryostatin 1, 26-succinylbryostatin 1 was unable to cause aggregation of human platelets. Although injection of bryostatin-1 into mice carrying B16 melanoma inhibits tumor growth, there was no significant inhibition of melanoma growth when identical doses of 26-succinylbryostatin 1 were injected. Therefore, 26-succinylbryostatin 1 shares some but not all of the pharmacologic properities of bryostatin 1. This compound can activate protein phosphorylation without lowering cytosolic levels of PKC.

Keywords: PKC; 26-Succinylbryostatin 1; Antitumor activity; PKC translocation

1. Introduction

Bryostatin 1, a naturally occurring macrocyclic lactone derived from a marine bryozoan, *Bugula neritina* [1] exhibits both in vitro and in vivo anticancer activity. The cellular basis for the antitumor effects of this compound is unknown; however, an initial event in the mechanism of action of bryostatin 1 is activation of PKC [2]. Treatment of cells with bryostatin 1 activates PKC stimulating phosphorylation of proteins, causing the translocation of PKC to the membrane and leading to the eventual degradation of PKC [3]. However, bryostatin 1 does not affect all PKC isoforms in an equivalent fashion [4]. Although it induces the translocation of α, δ, and ε isoforms, the α isoform is degraded much more rapidly [5].

In mice the administration of bryostatin 1 blocks the growth of P388 [6], B16 melanoma [7], and L10A B-cell lymphoma [8]. Although Bryostatin 1 inhibits clongenic growth of K562 cells (a myeloid cell line), REH cells (a pre B-lymphoblastic cell line), and fresh acute nonlymphocytic leukemia cells, it shows only mariginal activity against clonogenic CEM cells (a T-lymphoblastic cell line) [9]. Although, bryostatin 1 stimulates the activity of PKC, other potential mechanisms for the antitumor activity of this agent have been considered [10]. In humans bryostatin 1 stimulates the release of TNFα and IL-6, suggesting that cytokine release could play a role in this compound's antitumor activity [11]. Because, bryostatin 1 has been shown to trigger the development of cytotoxic T lymphocytes [12,13] , these expanded T lymphocytes have been used in a protocol of adoptive antitumor immunity [14].

Two phase I trials of this agent have been carried out in humans [11,15]. The major side effect and dose limiting

* Corresponding author. Fax: +1 (205) 9756911.

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toxicity of bryostatin 1 was muscle aches and joint pains which were not associated with any abnormality on EMG or elevation of creatinine phosphokinase. In addition, a transient and immediate fall in platelet and WBC count was also seen, but both of these levels returned to normal in a matter of days. The infusion of bryostatin 1 in 60% ethanol caused venous sclerosis, therefore the adminstration of bryostatin 1 in this vehicle was stopped. The compound was then delivered in polyethylene glycol, ethanol, and Tween 80 (PET 60/30/10 v/v). Six patients receiving this formulation experienced skin flushing, dyspnea, hypotension, and bradycardia which was thought to be secondary to the vehicle and not to bryostatin 1. Therefore, the lack of aqueous solubility of bryostatin 1 caused significant patient delivery problems.

In the process of developing antibodies to bryostatin 1, we synthesized the triethanolamine salt of 26-succinylbryostatin 1, which was found to have greatly increased solubility in aqueous solutions when compared to bryostatin 1. Because of the potential clinical utility of 26-succinylbryostatin 1, we have evaluated its biological activity in in vitro assays, after addition to cells in tissue culture, and in an animal tumor model. Our studies demonstrate that while in some tissue culture assays 26-succinylbryostatin 1 has similar biological activity to bryostatin 1, in animals bryostatin 1 is more potent than 26-succinylbryostatin 1 as an antitumor agent.

2. Materials and methods

2.1. Chemicals and reagents

Bryostatin 1 was provided by Dr. Kenneth Snader of the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Both bryostatin 1 and succinylbryostatin 1 were stored at −20°C in 100% DMSO. Both compounds were diluted to 0.1% DMSO upon addition to tissue culture medium. All control experiments were done with the addition of identical amounts of DMSO.

2.2. PKC assay and Western blot

U937 cells obtained from the ATCC were grown in DMEM medium containing 10% iron-supplemented bovine calf serum, antibiotics, and 4 mM glutamine in a 5% CO_2-humidified atmosphere. Cells were pelleted and washed twice with PBS. PKC assays were carried out as previously described using 100 μM [γ-^{32}P]ATP (~60 cpm/pmol) [2,3]. To assay membrane bound PKC, after treatment with 26-succinylbryostatin 1 or bryostatin 1, cells were pelleted, washed, and sonicated [2,3]. The sonicate was spun in an ultracentrifuge at 200000 × g for 30 min. The pellet was extracted with 1% Triton X-100, the extract placed over a DEAE-cellulose column, and PKC eluted as previously described [41]. Both the eluate and supernatant from the ultracentrifugation were assayed for PKC activity [2,3].

Western blots of PKC were done on both the supernatant and the pellet as described above. Approx. 300 μg of protein were loaded on a 8% SDS-PAGE gel and then transferred to nitrocellulose. Blots were blocked with 2% BSA in Tris-buffered saline, pH 7.6, for 1 h. These were then incubated with antiprotein kinase C antibody to PKC δ (Santa Cruz Biotechnology, Santa Cruz, CA) After incubation the western blot was washed with Tris-buffered saline (pH 7.6) containing (0.1% Tween-20) followed by incubation with a secondary antibody and the antigen/antibody complex detected by ECL (Amersham Life Sciences, Arlington Heights, Il).

2.3. Competition assay for PKC binding

A GST fusion protein containing amino acids 92–173 of PKC [GST-Cys2 (92–173)] (a gift of Dr. R. Bell) was grown and purified as described [16,17]. To evaluate the ability of bryostatin 1 and 26-succinylbryostatin 1 to inhibit the binding of [^{3}H]PDBu to this PKC fragment, the GST-Cys2 was first purified on glutathione beads, and then the protein was eluted with three volumes of 0.3 ml of 5 mM glutathione, 50 mM Hepes, pH 8.0, and 10% ethylene glycol. The eluted protein was combined, and the assay was carried out as previously described [16,17]. The assay mixture (100 μl) contained 2 mM $CaCl_2$, 40 μg/ml of sonicated phosphatidylserine (Avanti Polar Lipids, Birmingham, AL), 40 μl GST-Cys2 (approx. 20 μg), Hepes 20 mM, pH 7.8, 154 nM [^{3}H]PDBu, and bryostatin 1 or 26-succinylbryostatin 1 diluted in 100% DMSO. The reaction mixture was incubated for 10 min at room temp. The binding reaction was stopped by the addition of ice-cold buffer (20 mM Tris-HCL, pH 7.5, 200 μM $CaCl_2$, in 20% methanol). The mixture was filtered over glass fiber filters which were then washed with 10 ml of stop buffer. The filters were then counted in a scintillation counter.

2.4. Immunoprecipitation

U937 cells were labelled with 0.1 mCi/ml of [^{35}S]methionine in miminal essential medium contianing 10% (v/v) dialyzed bovine calf serum lacking methionine. Native lysates were prepared by lysis in radioimmunoprecipitation assay (RIPA) buffer [39]. Lysates were clarified by centrifugation, and the supernatant was precleared twice with protein A-sepharose beads. c-Jun was immunoprecipitated with rabbit antisera at 1:500 dilution. Immune complexes were collected with protein A-sepharose beads, washed extensively, and resolved on SDS, 10% polyacrylamide gels.

2.5. CAT assay

U937 cells in the logarithmic phase of growth were transfected by electroporation [18]. CAT assays were performed as described [19]. 48 h after treatment, cells were pelleted, washed twice with Dulbecco's phosphate-buffered saline, and suspended in 100 μl of a buffer containing 250 mM Tris-HCL, pH 7.8, 1 mM EDTA, 1 mM EGTA, and 2 μg aprotinin. Cells were freeze-thawed six times, and the homogenate was spun for 10 min in a microcentrifuge. The supernatant was removed, and the protein content was determined.

The amount of protein used in the CAT assay was normalized by cotransfecting 5.0 μg of a plasmid containing β-galactosidase was assayed using a reaction mixture containing 30 μg (1 μg/μl), 3 μl magnesium buffer (1 μl 1.0 M $MgCl_2$, 350 μl β-mercaptoethan ol, 550 μl water), 66 μl *O*-nitrophenyl-β-D-galactopyranoside (4 mg/ml dissolved in sodium phosphate buffer, pH 7.5). The mixture was incubated at 37°C for 30 min–24 h until light yellow color developed. The reaction was stopped with 500 μl of $NaCO_3$ and absorbance measured at 410 nm.

Protein from each time point was added to 200-μl reaction containing 250 mM Tris-HCL, pH 7.8, 0.5 mM acetylCoA, and [^{14} C]chloramphenicol (0.25 uCi). The reaction mixture was incubated for 1 h at 37°C and then extracted with 1 ml of ethyl acetate. The majority of the ethyl acetate was removed by centrifugation under vacuum, and the remaining material was spotted onto a silica thin layer plate. After chromatography in chloroform/methanol (95:1), the plate was dried, and autoradiography was done for 24 h.

2.6. Platelet function

Human platelet-rich plasma was incubated with varied amounts of bryostatin 1 or 26-succinylbryostatin 1, and lumiaggregometry [20,21] was performed. Aggregation of platelets is measured by the ability of platelets to scatter light. Absorbance is plotted over time on the *Y*-axis following the addition of the agonist.

2.7. Animal studies

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, 5% fetal bovine serum, L-glutamine, 2 mM, non-essential amino acids, 100 μM, sodium pyruvate, 1 mM, and antibiotics. Prior to injection into the mice, cells were trypsinized and washed 3 times with PBS; then the tumors were established by the injection of 1×10^6 cells/0.2 ml PBS into the tail vein. C3H/Hen mice were obtained from the Charles River Laboratories (Raleigh, NC). Three days after tumor injection, mice were begun on daily i.p. (1 μg/mouse of either bryostatin 1 or 26-succinylbryostatin 1) injections of different bryostatins in 0.2 ml of 10% DMSO/PBS for a total of 15 days. On day 18 after the injection of tumor cells, the animals were weighed, the lungs removed, and the left lung weighed. A section of the right lung was examined pathologically to document that the animal had been successfully engrafted with tumor cells.

2.8. Synthesis of 26-succinylbryostatin 1

To a solution of bryostatin 1 (23 mg, 25.4 μmol) in CH_2Cl_2 (1.0 ml) was added succinic anhydride (3.8 mg, 38 μmol, 1.5 equiv.) and dimethylaminopyridine (4.6 mg, 38 μmol, 1.5 equiv.). After stirring at ambient temperature for 18 h, the solvent was evaporated. The residue was purified by centrifugal thin layer chromatography on silica gel (5% CH_3OH/CH_2Cl_2 Æ 100% CH_3OH) to yield 23.7 mg (78%). The synthesis of the triethanolammonium bryostatin 1 26-succinate was accomplished by dissolving 0.53 mg (0.53 μmol) 26-succinylbryostatin 1 in 1.0 ml ethanol and adding 75 μl of an ethanol solution of triethaolamine (1.05 mg/ml, 0.079 mg, 0.53 μmol).

The structure of the compound was verified by NMR spectroscopy on a GE Omega 500 MHz spectrometer. Spectra were obtained in CD_2Cl_2, and chemical shifts were assigned by ^{1}H-COSY (two dimensional proton-proton COrrelated SpectroscopY) and ^{1}H-^{13}C HMQC (Heteronuclear Multiple Quantum Coherence) NMR experiments and comparison to published spectra [22]. After stirring at ambient temperature for 10 min the solvent was evaporated to yield a white solid residue.

To determine the relative solubilities of bryostatin 1 and 26-succinylbryostatin 1triethanolamine salt, each compound (0.53–0.55 μmol) in a methanol stock solution was added to separate 10 ml pear flasks, and solvent was evaporated under reduced pressure [23]. To each flask was added 1.5 ml of 1-octanol-saturated water, followed immediately by addition of 1.5 ml water-saturated 1-octanol. The biphasic solutions were mixed vigorously with a Teflon stir vane for 10 min at ambient temperature (~ 23°C), and the solutions were transferred to 15 ml polypropylene tubes and centrigued at $1600 \times g$ for 15 min. The 1- octanol and water layers were assayed for absorbance at 263 nm using a Beckman DU-7 spectrophotometer. Bryostatin concentrations were calculated using a molar extinction coefficient (ϵ_{263}) of 28 700 for bryostatin 1 and 26-succinylbryostatin 1 triethanolamine salt [1].

3. Results

Using the method described above, the logarithm of the partition coefficient (log *P*) was found to be 2.88 for bryostatin 1, compared to 0.88 for the triethanolamine salt

of 26-succinyl bryostatin 1, indicating that the modified bryostatin 1 is 100-fold more water soluble than the parent compound.

Homogenates of U937 cells were used as a source of PKC to evaluate the ability of 26-succinylbryostatin-1 to activate PKC in vitro. The PKC assay was carried out in the presence of 6 μg/ml of phosphatidylserine and 1 mM $CaCl_2$ using histone IIIs as a phosphotransferase acceptor. In the absence of added bryostatins approximately 80 cpm/μg of cellular homogenate used as a source of enzyme were incorporated into the substrate (Fig. 1) . The addition of either 26-succinylbryostatin 1 or bryostatin 1 stimulated a concentration-dependent increase in PKC activity. Between 1–100 nM, bryostatin 1 stimulated a significantly greater PKC activity than identical concentrations of 26-succinylbryostatin 1 (Fig. 1) . However, at the maximal concentration tested, 1000 nM, there was no difference in the ability of these compounds to stimulate PKC activity in vitro (Fig. 1).

A competition assay was devised to examine the ability of 26- succinylbryostatin 1 to bind to PKC. Amino acids 72–173 of PKCγ bind phorbol esters. This short segment of the protein was expressed in bacteria as a fusion protein with glutathione S-transferase (GST) [16,17]. After expression, this fusion protein was purified from the bacterial homogenate by binding to glutathione-Sepharose beads, followed by elution with glutathione. Since the bryostatins and phorbol esters bind to similar, if not identical, sites on PKC, the ability of varied concentrations of 26-succinylbryostatin 1 or bryostatin 1 to bind to PKC could be measured by the displacement of [^{3}H]phorbol dibutyrate ([^{3}H]PDBu) from the GST-PKC fusion protein. Both bryostatin 1 and 26-succinylbryostatin 1 stimulated a dose-dependent decrease in the ability of [^{3}H]PDBu to bind to the GST-PKC protein (Fig. 2). However, at each concentration tested bryostatin 1 displaced more [^{3}H]PDBu than 26-succinylbryostatin 1 (Fig. 2). At 1000 nM bryostatin 1 competed for all of the sites to the [^{3}H]PDBu was bound; whereas, at the identical concentration of 26-succinylbryostatin 1 only approximately 35% of the [^{3}H]PDBu was displaced (Fig. 2).

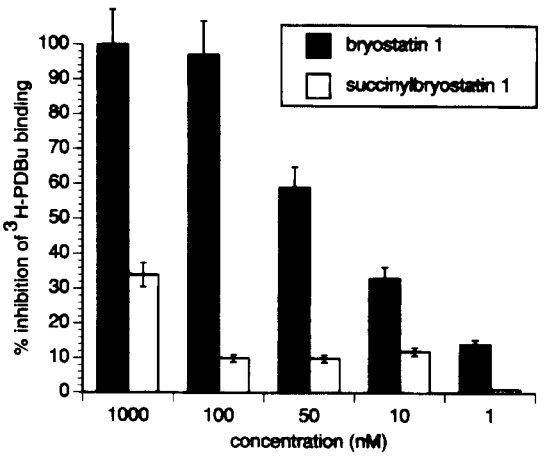

Fig. 2. Displacement of [^{3}H]PDBu from PKC by 26-succinylbryostatin 1. Varying concentrations of either 26- succinylbryostatin 1 or bryostatin 1 were added to a reaction containing GST-Cys2 (92-173), $CaCl_2$, and phosphatidylserine for 10 min at room temperature. The reaction was diluted with buffer (see Section 2) and filtered. The reaction was done in triplicate, and the average of duplicate experiments as well as the S.E. is shown.

To examine whether 26-succinylbryostatin 1 was able to cross the cell membrane and act as a biologic effector in vivo, the effect of 26-succinylbryostatin 1 on U937 human

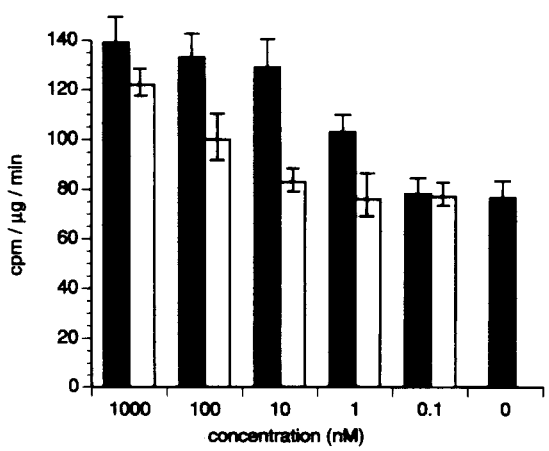

Fig. 1. Activation of PKC by 26-succinylbryostatin 1. U937 cells were lysed and the homogenate ultracentrifuged for 30 min at 200000× *g*. The supernatant was used as a source of PKC. Varying concentrations of 26-succinylbryostatin 1 (light bars) or bryostatin 1 (dark bars) were added to the reaction (see Section 2) which contained 6 μg/ml of phosphatidylserine (Avanti) and no diacylglycerol. After 5 min the reaction was stopped and filtered. The reaction was done in triplicate and the average of duplicate experiments as well as the S.E. is shown.

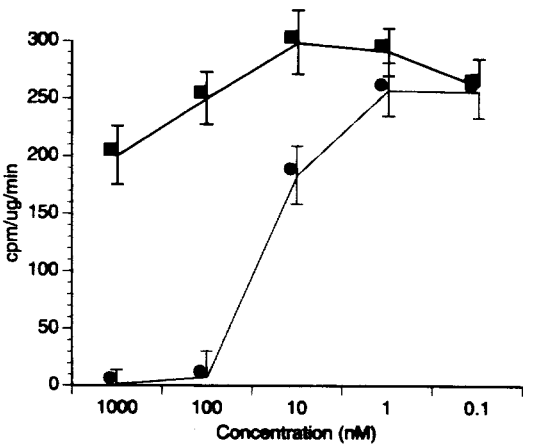

Fig. 3. Cytosolic PKC activity after treatment of U937 cells with 26-succinylbryostatin 1. U937 cells ($1.0 \cdot 10^7$) were treated with various concentrations of bryostatin 1 (light line) or 26-succinylbryostatin 1 (dark line) for 1 h. The cells were, pelleted washed, homogenized, and centrifuged at 200000× *g* for 30 min. 40 μg of cytosolic protein were assayed for 5 min as described in Section 2. The results are the average of three experiments done in triplicate, and the S.E. of these measurements is shown.

leukemic cells was examined. The addition of bryostatin 1 to U937 human leukemic cells stimulates the translocation of PKC from the cytosol to the membrane. This translocation causes a drop in measurable cytosolic PKC activity and an increased association with the plasma membrane. To determine whether 26-succinylbryostatin 1 is able to affect the cytosolic activity of PKC, U937 cells were treated with varying doses of either bryostatin 1 or 26-succinylbryostatin 1 for 1 h. The cells were then broken open and the cytosolic fraction separated from membranes by ultracentrifugation. The cytosolic PKC activity was then measured. The addition of bryostatin 1 to these cells stimulated a concentration-dependent decrease in the cytosolic PKC activity (Fig. 3). In contrast, given the variability of the assay, no significant change in the cytosolic activity of PKC was seen after treatment with 26-succinylbryostatin 1. To examine whether succinylbryostatin 1 stimulated tranlocation of PKC to the particulate fraction, the cell pellet was extracted with Triton X-100 and the PKC activity measured in both fractions (Fig. 4A). In a

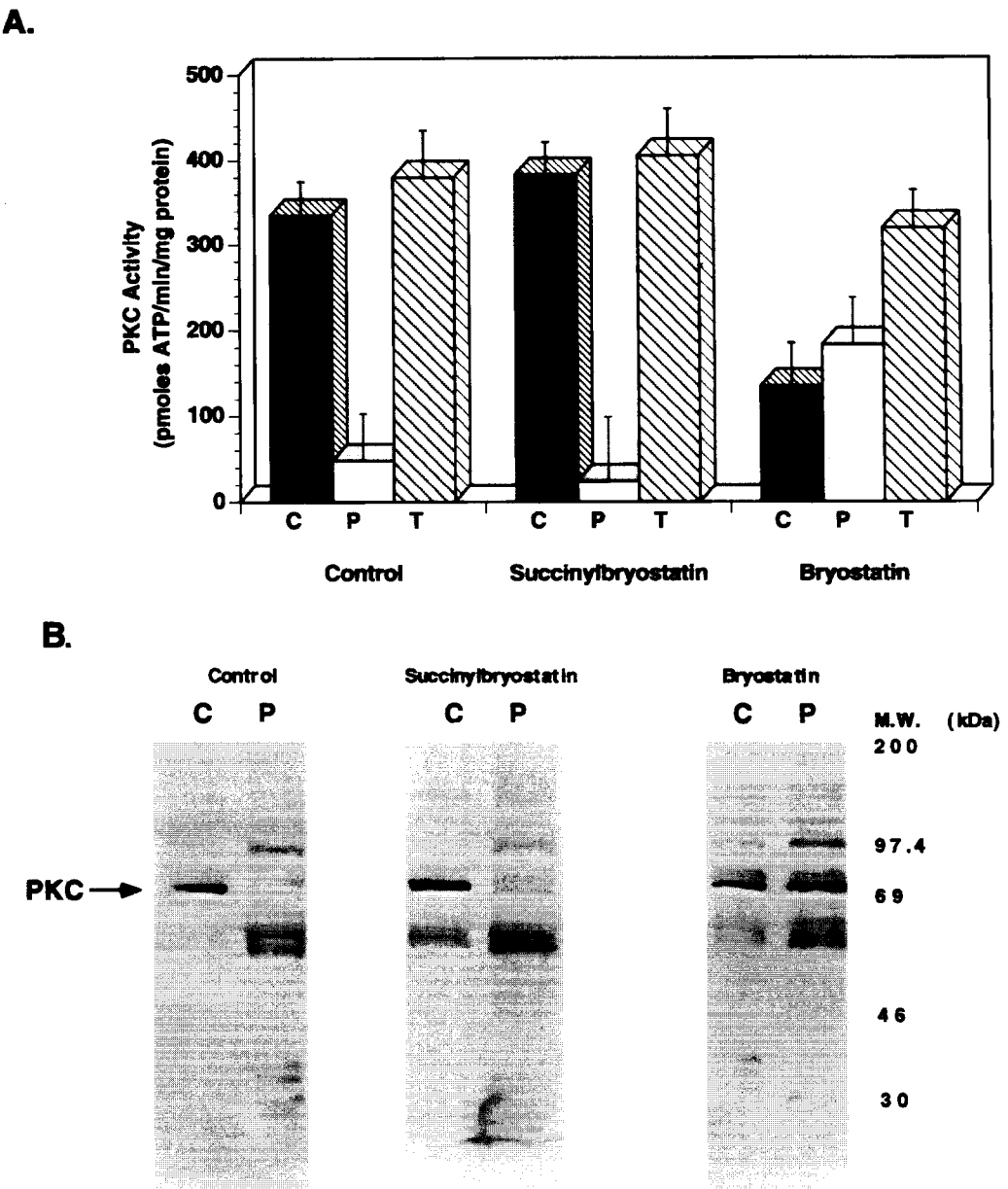

Fig. 4. Membrane association of PKC in bryostatin 1 and 26-succinylbryostatin 1 treated cells. (a) PKC activity in the cytosol (C) and pellet (P). $5 \cdot 10^7$ U937 cells were treated with either DMSO (control), 26 succinylbryostatin 1 (0.1 μM), or bryostatin 1 (0.1 μM) for 30 min. The cytosolic and membrane bound forms were assayed as described in Sections 2. The experiment was done in duplicate with each point representing the average of 6 values. The standard deviation of these values for the cytosol (C), pellet (P) and total (T) is shown. (b) PKC western blot of U937 cells. $2 \cdot 10^7$ U937 cells were treated with bryostatin 1, 26-succinylbryostatin 1 or vehicle for 30 min. The amount of PKC δ in the cytosol (C) and pellet (P) fractions was evaluated by western blot as described in Section 2. The molecular weight (M.W.) standards are shown in kDa.

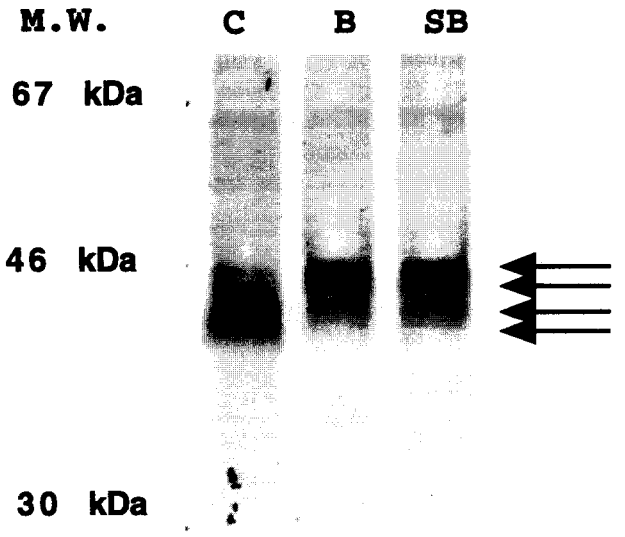

Fig. 5. 26-Succinylbryostatin 1 stimulates c-Jun phosphorylation. $1 \cdot 10^7$ U937 cells were labelled with [^{35}S]methionine (100 μCi/ml) for 4 h followed by treatment with 1 μM 26-succinylbryostatin 1 (SB) or bryostatin 1 (B) for 30 min. The cells were then pelleted and homogenized, and c-Jun immunoprecipitation was carried out [18]. The arrows denote bands which are retarded secondary to phosphorylation.

separate experiment, the pellet (P) and cytosol (C) fractions from bryostatin 1 and 26-succinylbryostatin 1 treated U937 cells were run on an SDS-PAGE and Western blotted with an antibody specific for PKC delta (δ) (Fig. 4B). In both assays bryostatin 1 translocated PKC to the membrane while 26-succinylbryostatin 1 did not as evidenced by modulation of enzyme activity and PKC δ location. These data could be interpreted to mean that because of increased water solubility, 26-succinylbryostatin 1 did not enter the cell; or, that because of decreased lipid solubility of 26-succinylbryostatin 1, no or little stimulation of PKC translocation took place.

To evaluate further whether 26-succinylbryostatin 1 had any biologic effects on these cells, the ability of this compound to stimulate the phosphorylation of c-Jun protein was evaluated. c-Jun is a protein which is capable of dimerizing through its leucine zipper [24]. As a homodimer or a heterodimer with the c-fos protein, it binds to DNA upstream of the start site of transcription and acts to enhance transcription [25]. The addition of PKC activators, including phorbol esters and bryostatin 1, to U937 cells stimulates the activity of a protein kinase which phosphorylates the c-Jun (identified as c-JATPK [26], JNK [27], SAPK [28]). Phosphorylation on a number of serines causes the c-Jun protein to migrate more slowly upon SDS-PAGE electrophoresis giving the formation of 3 retarded bands [18]. To examine the ability of 26-succinylbryostatin 1 to stimulate phosphorylation of c-Jun, U937 cells were labelled with [^{35}S]methionine and then treated for 30 min with either 1000 nM 26-succinylbryostatin 1 or bryostatin 1, and the c-Jun was then immunoprecipitated. The immunoprecipitate was run on an SDS-PAGE, and the gel was dried and flurographed (Fig. 5). Both bryostatin 1 and 26-succinylbryostatin 1 stimulated phosphorylation of c-Jun, as demonstrated by the retarded mobility of c-Jun bands.

To establish a dose-response curve for the biologic effects of 26-succinylbryostatin 1, the effect of this compound on AP-1 mediated transcription was evaluated. c-Jun as a homodimer or a heterodimer with members of the Fos family of proteins binds to the DNA sequence 5′-TCAGTCA-3′ [29]. Phosphorylation of c-Jun enhances transcription from this DNA sequence [18]. To evaluate the ability of 26-succinylbryostatin 1 to stimulate transcription from this sequence, U937 cells were transfected with a cDNA containing five copies of the AP-1 enhancer upstream of a chloroamphemicol acetyltransferase (CAT) reporter gene. These cells were then treated for 72 h with varying concentrations of either 26-succinylbryostatin 1 or

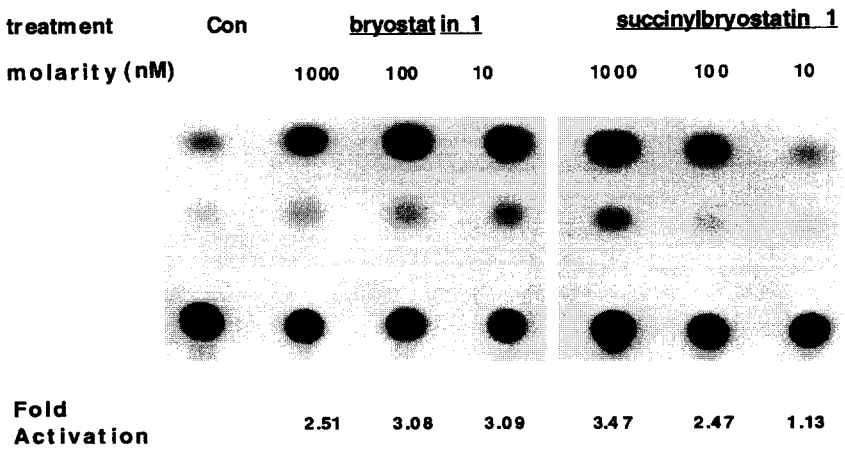

Fig. 6. 26-Succinylbryostatin stimulates 5 × -AP-1 activity. $7 \cdot 10^7$ U937 cells were electroporated with 70 μg of 5 × -AP-1 CAT cDNA (19). 24 h later the cells were divided ($1 \cdot 10^7$cells/aliquot) and each aliquot was treated for 72 h with various concentrations of 26-succinylbryostatin 1 and bryostatin 1. At the end of 72 h the cells were homogenized, and CAT assays were performed as described in Section 2. The modified chloramphenicol was scraped from the TLC plate and counted. The fold-activation is calculated by dividing the control value into those from bryostatin-treated cells.

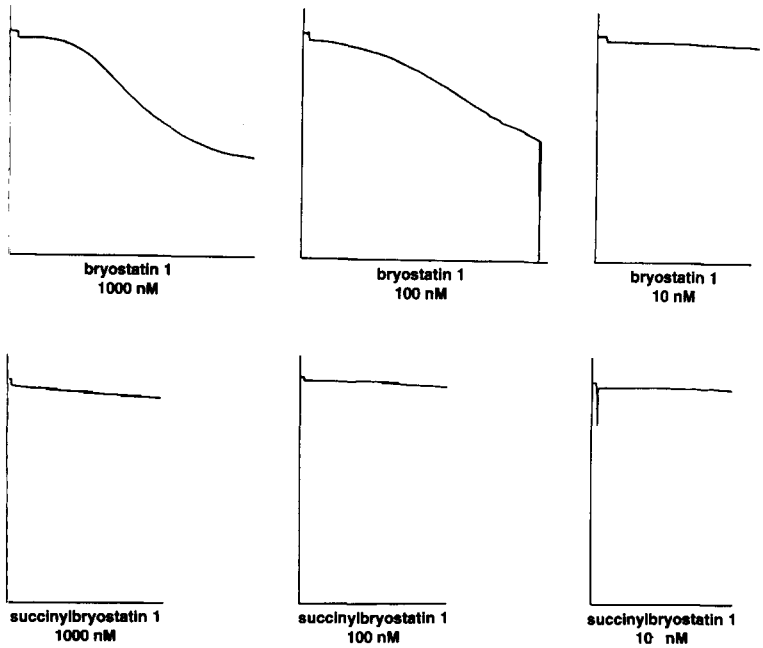

Fig. 7. Lack of platelet aggregation after treatment with 26-succinylbryostatin 1. Human platelet-rich plasma was incubated with varied concentrations of 26-succinylbryostatin 1 and bryostatin 1. The aggregation of platelets is measured by the ability of platelets to scatter light. Absorbance is plotted over time on the *Y*-axis following the addition of the agonist.

bryostatin 1 (Fig. 6). The cells were then lysed, a β-galactosidase assay done to control for transfection efficiency, and the CAT activity was measured. The addition of 1000 nM 26-succinylbryostatin 1 and bryostatin 1 stimulated 5 × -AP-1 enhancer activity (Fig. 6). There was a dose-response relationship for the CAT activity stimulated by 26-succinylbryostatin 1 with 10 nM showing little stimulation above background. In contrast, even at 10 nM, bryostatin 1 was still active at stimulated 5 × -AP-1 activity (Fig. 6). Together the results on c-Jun phosphorylation and 5 × -AP-1 driven CAT activity suggest that 26-succinylbryostatin 1 is capable of entering cells and stimulating biologic events.

The addition of bryostatin 1 to U937 cells induces an inhibition of cell growth and differentiation of these cells to monocyte/macrophages as evidenced by the induction of monocyte specific enzymes [40]. To evaluate the effect of 26-succinylbryostatin 1, U937 cells were grown in the presence of this compound for 96 h. Cell growth was measured and cells were stained for α-napthylesterase, a marker of monocyte/macrophage differentiation. 1 μM 26-succinylbryostatin 1 induced a 60% inhibition of cell growth (average of triplicate determinations in two experiments), whereas the 0.1 μM treatment caused a 20% inhibition of cell growth and 0.01 μM had no effect. The induction of α-napthylesterase paralleled the inhibition of cell growth with 66% of cells staining enzyme positive after treatment with 1 μM 26-succinylbryostatin 1. Thus, the concentrations of 26-succinylbryostatin 1 which activate gene transcription also inhibit cell growth and induce increases in an enzyme found in differentiated leukemic cells.

When blood is drawn from mice injected i.v. with bryostatin 1 the platelets contained in these blood samples cannot be activated [30]. This inability to activate the platelets is secondary to the bryostatin 1-induced activation which occurs in vivo. Also, in vitro the addition of bryostatin 1 directly to human platelets causes the aggregation of these platelets, making them no longer responsive to additional stimuli [30]. To examine the effect of 26-succinylbryostatin 1 on human platelets, varying doses of this compound were added directly to human platelets, which were then subjected to lumiaggregometry. Aggregation of the platelets is evident after addition of 1000 and 100 nM

Table 1
The effect of bryostatin 1 and 26-succinylbryostatin 1 on tumor regression and weight loss

	Control [a] ($n = 10$)	Bryostatin 1 treatment [b] ($n = 10$)	Succinyl-brostatin [c] ($n = 10$)	Uninjected ($n = 8$)
Lung weights (g)	0.327 ± .045 [d]	0.205 ± .025 [e]	0.262 ± .041 [f]	0.07 ± 0.006

[a] Mice were injected with 10^6 melanoma cells received no treatment and were sacrificed on day 18.
[b] Mice were injected with melanoma cells plus 1 μg bryostatin 1 i.p. from day 3–17.
[c] Mice were treated with melanoma cells plus 1 μg of 26-succinylbryostatin 1 i.p. from day 3–17.
[d] Values shown are the mean ± S.E.
[e] Different from control at $P < 0.0327$.
[f] Different from control at $P < 0.2408$.

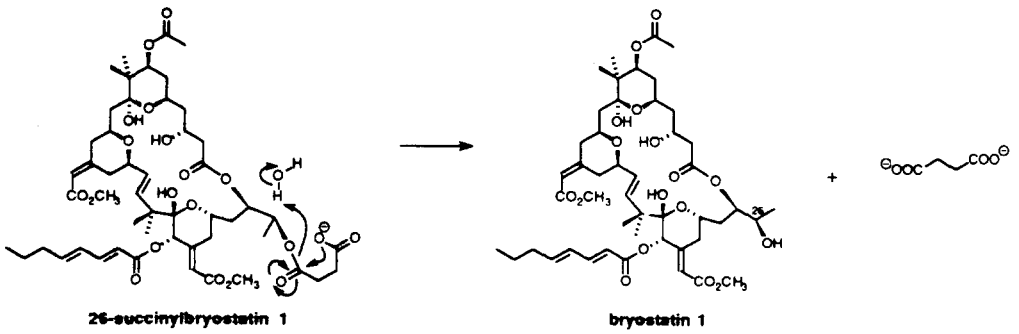

Fig. 8. A potential mechanism for intramolecular hydrolysis of 26-succinylbryostatin 1. A potential mechanism for the intramolecular hydrolysis of 26-succinylbryostatin 1 is diagramed.

bryostatin 1 but is not apparent after treatment with similar doses of 26-succinylbryostatin 1 (Fig. 7). Although there are biologic effects of these doses in human cells in culture, no significant aggregation of human platelets was evident.

Bryostatin 1 injection into mice previously injected intravenously with B16 melanoma both decreases the growth of pulmonary melanoma and prolongs the life of the mice [7,31]. This decrease in tumor growth in the lungs correlates directly with post-mortem lung weight. To evaluate whether 26-succinylbryostatin 1 could act as an anticancer compound, mice were injected intravenously with B16 melanoma cells. Three days later the mice were injected intraperitoneally with 1 μg bryostatin 1 or 26-succinylbryostatin 1, and these injections were continued daily for 15 days. The bryostatin 1 dose was chosen, because when given at higher doses on a daily basis bryostatin 1 can be lethal (data not shown). After treatment was concluded, the mice were then sacrificed, and the lung weights were recorded. Because melanoma growth was localized in the lung, measurement of the weight of this tissue was chosen as an endpoint rather than animal mortality. As reported previously [7,31], bryostatin 1 signficantly inhibited the growth of the melanoma in the lungs when compared to untreated tumor bearing animals (Table 1). While the 26-succinylbryostatin 1 injected animals showed decreased lung weights, when the variability among the animals was taken into account the decrease in lung weights was not significant (Table 1). The lung weights for bryostatin 1 injected animals were significantly different from uninjected animals, suggesting that although bryostatin 1 inhibited tumor growth it did not completely irradicate the melanoma cells.

4. Discussion

A significant number of patient side effects have been caused by the vehicle in which bryostatin 1 is administered. Thus, modifications of the bryostatin 1 ring might increase the solubility of this compound in aqueous solutions and allow this modified bryostatin to be administered in physiologic solutions. The bryostatin 1 molecule displays a limited number of functional groups which might be utilized for preparing prodrug derivatives. The C-26 alcohol moiety, however, may be readily esterified [32]. Alcohol moieties have often been derivatized with succinate esters to increase the water solubility of lipophilic drugs [33]. For example, the free acid of 2′-succinyl taxol was prepared [34], but this compound had disappointing in vivo activity against P388 leukemia cells when compared to taxol [7]. Interestingly the antitumor activity of 2′-succinyl taxol against B-16 melanoma xenografts was partly dependent on the counterion employed [35]. The 2′-succinyl taxol triethanolamine salt was more active than the parent drug, taxol, and either the 2′-succinyl free acid or sodium salt derivatives. We have synthesized the triethanolamine salt of 26-succinylbryostatin 1, and have evaluated its biological activity because of (1) its increased solubility in aqueous solutions, [2] its potential to undergo an intramolecular catalytic release of bryostatin 1 (Fig. 8), and [3] the possibility that this agent could be acted upon by plasma or tissue esterases to liberate bryostatin 1. To examine the possibility that 26-succinylbryostatin 1 was cleaved or went through intramolecular catalytic release, 26-succinylbryostatin 1 was incubated with U937 cells or alone in medium for 72 h. The presence of 26-succinylbryostatin 1 breakdown products was then examined by HPLC followed by mass spectrometry. By this method, only 26-succinylbryostatin 1 and no altered compounds was found in the medium when incubated with or without cells. The levels of 26-succinylbryostatin 1 appeared grossly unchanged. Thus, although the chemical structure suggests the possibility of an intramolecular rearrangement, none could be detected in tissue culture. Insufficient amounts of compound were available to analyze structural changes in 26-succinylbryostatin 1 in mice.

We have used a fusion protein expressing the phorbol ester-binding domain of PKC bound to GST to examine the ability of 26-succinylbryostatin 1 to bind to PKC. We find that even when 26-succinylbryostatin 1 is present in 10-fold excess (1 μM) of [^{3}H]PDBu, it displaces only ~ 30% of the PDBu, in comparison to bryostatin 1 displaces 100%. The decreased binding of 26 substituted

bryostatins to PKC has been previously demonstrated [36]. Esterification of the 26 position of bryostatin 4 caused a dramatic decrease in affinity for PKC [36], suggesting that this position was important for PKC binding. This decrease in ability to bind PKC is reflected in the decreased ability of 26-succinylbryostatin 1 to stimulate PKC-mediated phosphorylation of the histone substrate (Fig. 1). In comparison, when examining the bryostatin stimulation of PKC phosphorylation at the highest concentration tested (1 μM), the activity of these two bryostatins differed little, suggesting that stimulation of PKC phosphorylation may be less sensitive to differences in these two compounds than PKC binding affinity.

The addition of bryostatin 1 to both NIH-3T3 [37] and U937 cells causes translocation from the cytosol to the membrane of a number of the PKC isoforms. This translocation is associated with a drop in cytosolic PKC. However, in comparison to bryostatin 1, 26-succinylbryostatin 1 did not stimulate significant loss of cytosolic PKC activity nor association with the membrane. Western blot using an antibody to PKC δ, a novel PKC isoform, also did not disclose any translocation. A longer incubation (4 h) with 26-succinylbryostatin 1 gave identical results, suggesting that more prolonged exposure did not increase the amount of this compound that entered the cell. These findings could result from either 26-succinylbryostatin 1 not entering the cell or, because of its decreased lipid solubility, not binding PKC on the membrane.

Because PKC can be activated by bryostatin 1 in the absence of membrane translocation [38], it was possible that 1 μM 26-succinylbryostatin 1 stimulated PKC in cells without significant membrane translocation. To evaluate this possibility both the phosphorylation of c-Jun protein and the activation of transcription by this protein were measured. Like bryostatin 1, 26-succinylbryostatin 1, 1000 and 100 nM stimulated down-stream protein kinases to phosphorylate c-Jun and the activation of transcription from a 5 × AP-1 enhancer element. The biological activity of 10 nM 26-succinylbryostatin 1 was not markedly different from control untreated cells. Measurements of growth suggest that concentrations that have an effect on transcription also inhibit cell growth, suggesting that at these doses 26-succinylbryostatin 1 has biologic activity in tissue culture.

A potential major side effect of bryostatin 1 is platelet activation. In human trials a drop in platelet count has been seen but no obvious bleeding was apparent [11,15]. Higher doses of bryostatin 1 have been shown to activate the aggregation of human platelets in vitro [30]. However, in comparison to bryostatin 1, 26-succinylbryostatin 1 did not stimulate any changes in platelet function. It is possible that 26-succinylbryostatin 1 was not sufficiently lipid soluble to enter the platelet or that PKC translocation was necessary for platelet aggregation.

Murine B16 melanoma when injected intravenously forms pulmonary colonies that have has proven responsive to bryostatin 1 [7,31]. After 26-succinylbryostatin 1 injection, although there was a trend towards inhibition of melanoma growth in the lungs with lower lung weights than controls (0.262 vs. 0.327), this effect was not statistically significant when the variability of the weights was taken into account. It is possible that the high lipid solubility of bryostatin 1 while making it difficult to administer the compound is necessary for the antitumor effects of this compound. Also, it is possible that the acqueous solubility of 26-succinylbryostatin 1 could lead to the rapid excretion of this compound.

Our work suggests that 26-succinylbryostatin 1 does not bind as tightly to PKC as PDBu but is capable of activating PKC to phosphorylate histone. In tissue culture 26-succinylbryostatin 1 stimulates the phosphorylation of specific proteins without marked translocation of PKC to the membrane and inhibits the growth of leukemic cells. Since it is unknown how bryostatins inhibit tumor growth, the importance of these in vitro observation to the mouse melanoma model is unclear. Further testing of modified bryostatins with higher aqueous solubility combined with the potential to break down to bryostatin 1 in vivo seems warranted to develop better chemotherapeutic agents and to clarify whether lipid solubility is necessary for chemotherapeutic effect.

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