The Bryostatins Inhibit Growth of B16/F10 Melanoma Cells in Vitro through a Protein Kinase C-independent Mechanism: Dissociation of Activities Using 26-Epi-Bryostatin 1

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ABSTRACT

Bryostatin 1 is a potential cancer chemotherapeutic agent in Phase II clinical trials, with positive responses observed for malignant melanoma, among other tumors. The bryostatins are known to be potent ligands for protein kinase C (PKC), functioning as partial antagonists. In the present study, we explore the mechanism by which the bryostatins inhibit growth of B16/F10 mouse melanoma cells in vitro. Three experimental approaches suggest that the growth inhibition is independent of PKC. First, we characterized in detail the translocation and down-regulation of the PKC isoforms α, δ, and ε in response to phorbol ester and bryostatin 1 in these cells. Although the dose-response curves obtained for the translocation-activation of PKC isoforms showed good correlation with the growth-enhancing activity of phorbol 12-myristate 13-acetate, for no PKC isoform was there a good correlation with the growth-inhibitory activity of bryostatin 1. Second, inhibition of PKC enzymatic activity by the specific PKC inhibitor bisindolyl-maleimide I did not block the inhibition of thymidine incorporation induced by bryostatin 1. Finally, 26-epi-bryostatin 1, a stereoisomer of the naturally occurring bryostatin 1 designed to have markedly reduced affinity for PKC, inhibited the growth of the B16/F10 melanoma cell lines with potency similar to that of bryostatin 1. We confirmed here that 26-epi-bryostatin 1 showed 60-fold reduced affinity for PKC and 30–60-fold reduced potency to translocate and down-regulate PKC isoforms compared with bryostatin 1. We presume that the principal toxicity of bryostatin 1 reflects its interaction with PKC, and we would thus predict that epi-bryostatin 1 would be less toxic. Indeed, we found at least 10-fold reduced toxicity of 26-epi-bryostatin 1 in C57BL/6 mice compared with bryostatin 1. We conclude that the growth inhibition of the bryostatins, at least in this system, does not result from interaction with PKC. As exemplified by 26-epi-bryostatin 1, this insight permits the design of analogues with comparable growth inhibition to bryostatin 1 but with reduced toxicity.

INTRODUCTION

Malignant melanoma is one of the most rapidly increasing forms of cancer in the United States, with a 5-year survival rate of less than 50% for disease recognized at an advanced stage (1). Bryostatin 1 is a macrocyclic lactone isolated from Bugula neritina that has shown promising antitumor activity in both animal models and clinical trials (2–5). As with most chemotherapeutic agents, the maximal dosage of bryostatin 1 is limited by toxicity. In mice, bryostatin 1 doses greater than 200 μg/kg were lethal (3), whereas in humans, three bryostatin 1 infusions of 2 μg/kg caused significant side effects, including myalgia and hypotension (4).

In this report, we have explored the mechanism by which bryostatin 1 inhibits cell growth in B16/F10 melanoma cells in vitro. Bryostatin 1 is known to be a potent ligand for PKC (6, 7). In vitro, it activates the individual PKC isozymes to an extent similar to that of the typical phorbol esters (8). Biologically, however, bryostatin 1 functions as a partial antagonist for a subset of phorbol ester-induced responses (9). Multiple mechanistic differences may contribute to its unique spectrum of biological response, including selectivity in intact cells for the novel PKC isozymes δ and ε (10), slow kinetics of PKC translocation, and protection of PKC-δ from down-regulation at high bryostatin doses (10, 11). Both the protection of PKC-δ (10) and, in earlier studies, the antagonism of phorbol ester action on Friend erythroleukemia cell differentiation (12) were noncompetitive between bryostatin 1 and phorbol ester, consistent with the antagonism being mediated by a target not recognized with high affinity by the phorbol esters. A similar conclusion had been suggested indirectly by comparison of the extent of arachidonic acid release induced in C3H10T½ cells by bryostatin analogues and phorbol esters (13). Because the inhibition of melanoma cell growth is induced by bryostatin 1 at relatively high doses, in the present study we sought to explore whether this response could also be mediated through a non-PKC target.

If some subset of the actions of bryostatin 1 is mediated through a target other than PKC, then the structure-activity relationships for those responses should be different from those mediated by PKC. The structure-activity relationships for the phorbol esters have been extensively examined both for PKC binding (14) and for biological responses (15–17). Our detailed understanding of the phorbol ester pharmacophore has been further extended both by X-ray crystallography of the complex between phorbol ester and the PKC phorbol ester-binding domain (18) and by computer modeling using the nuclear magnetic resonance-defined structure of the phorbol ester-binding domain in PKC.2 These approaches uniformly emphasize the central role of the C20 hydroxyl group of the phorbol ester for the binding interaction. Computer comparison of bryostatin 1 with the phorbol esters indicates that the C26 hydroxyl group of bryostatin 1 corresponds to the C20 hydroxyl group of the phorbol ester (19, 20). This assignment is supported by the decreased binding activity for PKC of either the C26 acetate ester of bryostatin 1 (20) or the C26 epimer of bryostatin 4 (21). In the present study, this diminished activity of the C26 epimer of bryostatin 1 has provided a powerful tool to evaluate the mechanism of growth inhibition by bryostatin 1 in the B16/F10 melanoma cells. We report here that the C26 epimer of bryostatin 1 is equally potent to bryostatin 1 for inhibition of melanoma cell growth in vitro but has at least 10-fold reduced toxicity for mice.

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1 The abbreviations used are: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; BIM-I, bisindolyl-maleimide I.


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MATERIALS AND METHODS

Bryostatin 1 was isolated from B. neritina as described (22). 26-Epi-bryostatin was synthesized from bryostatin 1 and separated from its natural stereoisomer as previously described (23). PMA and BIM-1 (GF-109203X) were purchased from LC Laboratories (Woburn, MA). All chemicals were dissolved in DMSO.

The B16/F10 melanoma cell line with high pulmonary metastatic potency (provided by the National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD) was cultured as described previously (24).

For analysis of cell growth, melanoma cells were plated at a density of $10^3$ cells/well in Costar 12-well tissue culture plates on day 0. The cells were treated with different concentrations of bryostatin 1, 26-epi-bryostatin 1, or PMA dissolved in DMSO (final concentration, 0.1%) on days 1 and 3. Total cell numbers were determined on day 4 with a Coulter counter. For determination of $[^{3}H]$thymidine incorporation, melanoma cells growing in log phase at a density of 40-50% confluency were used. After treatment for the time period and with the agents as indicated, the cells were pulsed with 1 $\mu$Ci $[^{3}H]$thymidine (25 Ci/mmol; Amersham) for 4 h. The cells were harvested, and the incorporated radioactivity was counted with a Pharmacia Biotech 1218 scintillation counter.

The dissociation constants of 26-epi-bryostatin 1 and bryostatin 1 for recombinant, baculovirus-expressed PKC-α were measured as described (21). Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at the age of 8–10 weeks. Different doses of bryostatin 1 and 26-epi-bryostatin 1 were injected i.p. in a volume of 0.1 ml PBS containing 1% DMSO. To prevent unnecessary suffering, animals were sacrificed on appearance of symptoms of severe toxicity, as evidenced by significant weight loss (more than 10%), dehydration, and drop in body temperature.

For Western blot analysis of PKC isozymes, melanoma cells were treated at 50% confluency for either 10 min or 24 h with different doses of bryostatin 1, 26-epi-bryostatin 1, or PMA. Cells were also subjected to the same chronic treatment schedule used to assess cell growth inhibition over a 4-day period. All compounds were applied in DMSO (0.1% final concentration). A Western blot analysis was performed as described previously (10). The primary antibodies used were a monoclonal antibody against the catalytic domain of PKC-α (Upstate Biotechnology, Lake Placid, NY), affinity-purified polyclonal antibodies against the C-terminal region of PKC-δ and PKC-ζ (Research and Diagnostics Antibodies, Berkeley, CA), and an affinity-purified polyclonal antibody against PKC-ε (Life Technologies, Inc., Gaithersburg, MD). The specificity and lack of cross-reactivity of the primary antibodies were demonstrated using cloned PKC isozymes expressed in the baculovirus system, as described previously (10). Densitometric quantitation of the PKC isozymes was performed as described previously (10).

PKC activity was assayed in cell lysates by measuring the incorporation of $[^{3}P]P$ from $[^{γ^{32}}P]ATP$ (Amersham) into acetylated myelin basic protein peptide 4–14 (Life Technologies) as described previously with minor modifications (25). The cells were treated at 60–75% confluency with bryostatin 1, 26-epi-bryostatin 1, and/or BIM-1 for 10 min. The cells were lysed by sonication in 20 mM Tris-HCl (pH 7.4) containing 5 mM EDTA and 20 μM leupeptin. Five μl cell lysate were added to 15 μl kinase buffer [20 mM HEPES (pH 7.5), 10 mM MgCl₂, 0.5 mM CaCl₂, 1 mM 4-[2-aminoethyl]benzenesulfonyl fluoride, 20 μM leupeptin, and 10 μg/ml aprotinin] containing 50 μM substrate and 2 μCi $[^{γ^{32}}P]ATP$ at a concentration of 50 μM ATP. The kinase reaction was incubated for 10 min at 30°C. The incorporated radioactivity was measured with a Pharmacia Biotech 1218 liquid scintillation counter.

RESULTS

$[^{3}H]$Thymidine Incorporation and Cell Proliferation. We determined the effect of bryostatin 1 and PMA on the proliferation of the B16/F10 melanoma cell line. Bryostatin 1 inhibited $[^{3}H]$thymidine incorporation, with an $ED_{50}$ of 600 ± 80 nM ($n = 3$) and a maximum inhibition of 60% (Fig. 1A). PMA showed no significant effect on the $[^{3}H]$thymidine incorporation tested over a dose range between 0.1 nM and 3 μM (data not shown), except for a slight increase (120 ± 20%) at 10 nM PMA. We also tested the effect on cell growth of chronic treatment with bryostatin 1 or PMA. Bryostatin 1 showed 75 ± 5% ($n = 3$) inhibition of cell growth after 4 days of treatment at the highest dose tested (3 μM); the $ED_{50}$ was 50 ± 15 nM ($n = 3$; Fig. 1B). The trypan blue exclusion test showed less than 1% cell death. In contrast to bryostatin 1, PMA increased the cell growth by 75 ± 10% ($n = 3$) at a dose of 10 nM; the dose-response curve was biphasic, with smaller effects at higher and lower applied doses (Fig. 1B).

In B16/F10 melanoma cells, the following PKC isozymes were detected by Western blotting: PKC-α, PKC-δ, PKC-ε and PKC-ζ (Figs. 2, 3, and 5). We tested the regulation (translocation and down-regulation) of each of these isoforms on treatment with bryostatin 1 and PMA. We wished to compare the dose-response curves for the specific PKC isoforms with those for cell growth inhibition or enhancement. Previously, we had shown that PKC activators differ in their kinetics for translocating and down-regulating individual PKC isoforms (10). In mouse keratinocytes, bryostatin 1 was very slow to translocate all three isoforms, PKC-α, -δ, and -ε. Rates of translocation could be expressed in terms of the ratio of the potencies of a PKC activator to remove PKC from the soluble fraction at short (5–10 min) and long times (6–24 h; Ref. 10). Accordingly, in the present study,
we measured the potencies of bryostatin 1 and PMA to remove the individual PKC isozymes from the soluble fraction at a short time point (10 min) and a long time point (24 h). We also measured both translocation and down-regulation of the PKC isozymes after the chronic (4-day) application of both agents.

PKC-α. Bryostatin 1 translocated PKC-α from the soluble fraction to both the Triton X-100-soluble and -insoluble particulate fractions (Fig. 2A), with ED₅₀ of 8 ± 1.1 nM at 10 min and 1.2 ± 0.3 nM at 24 h (Table 1). (In the interest of brevity, only the quantitated dose-response curves for PKC-α are illustrated. The quality of the data for PKC-α and PKC-ε was comparable.) This 7-fold shift in the dose-response curves is similar to that observed in keratinocytes (10).

PKC-δ. Bryostatin 1 translocated PKC-δ from the soluble to the Triton X-100-soluble and -insoluble particulate fraction (Fig. 3A), with ED₅₀ of 2 ± 0.4 nM at 10 min (Fig. 4A) and 0.09 ± 0.01 nM at 24 h, giving a 22-fold shift in the dose-response curves (Fig. 4A). As with keratinocytes and fibroblasts (10, 11), in the melanoma cells bryostatin 1 showed a biphasic dose-response curve for down-regulating PKC-δ (Fig. 3B), with maximal down-regulation at 0.1–1 nM after both 24 h and 4 days of treatment (Fig. 4B). Lower (1–10 pm) and higher (100 nM–1 μM) concentrations failed to down-regulate this isozyme. Unlike the previous systems examined (10, 11), for which PKC-δ was protected only to the extent (about 35%) that it was already present in the particulate fraction of untreated cells, in the melanoma cells, bryostatin 1 at high doses completely prevented PKC-δ loss. The PKC-δ protected from down-regulation by high doses of bryostatin 1 was localized both in the Triton X-100-soluble and -insoluble particulate fraction. At 4 days, PKC-δ was removed by bryostatin 1 from the soluble fraction, with an ED₅₀ of 0.02 ± 0.004 nM (Fig. 4A).

PMA translocated PKC-δ from the soluble to both the Triton X-100-soluble and -insoluble particulate fraction, with ED₅₀ of 33 ± 10 nM at 10 min and 4.5 ± 0.1 nM at 24 h (Fig. 4C). This shift of the dose-response curve, which contrasts with that for PKC-α, is very similar to the one detected in keratinocytes (10). PKC-δ was almost completely down-regulated at 24 h, with an ED₅₀ of 4 ± 1 nM (Figs. 3B and 4D). On the 4-day treatment schedule, PMA down-regulated PKC-δ, with an ED₅₀ of 0.8 ± 0.1 nM, and removed this isozyme from the soluble fraction, with an ED₅₀ of 2 ± 0.8 nM (Fig. 4, C and D).

PKC-ε. Bryostatin 1 translocated PKC-ε from the soluble to both the Triton X-100-soluble and -insoluble particulate fractions (Fig.

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**Table 1** Potency of PMA, bryostatin 1, and 26-epi-bryostatin 1 for translocating, removing from the soluble fraction, and down-regulating PKC isozymes α, δ, and ε in B16/F10 melanoma cells

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Time</th>
<th>PMA</th>
<th>Bryostatin 1</th>
<th>26-Epi-bryostatin 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC-α</td>
<td>10 min</td>
<td>1 ± 0.5</td>
<td>8 ± 1.1</td>
<td>480 ± 150</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>6 ± 1.0</td>
<td>1.2 ± 0.3</td>
<td>26 ± 7</td>
</tr>
<tr>
<td></td>
<td>4 days</td>
<td>4 ± 0.7</td>
<td>0.06 ± 0.004</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>PKC-δ</td>
<td>10 min</td>
<td>33 ± 10</td>
<td>2 ± 0.4</td>
<td>39 ± 5</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>4.5 ± 0.1</td>
<td>0.09 ± 0.01</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>4 days</td>
<td>2 ± 0.8</td>
<td>0.02 ± 0.004</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>PKC-ε</td>
<td>10 min</td>
<td>0.7 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>26 ± 6</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>8 ± 1</td>
<td>0.04 ± 0.007</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>4 days</td>
<td>10 ± 2</td>
<td>0.02 ± 0.008</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

**Table 2** ED₅₀ for translocation or removal from the soluble fraction (nM)

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Time</th>
<th>PMA</th>
<th>Bryostatin 1</th>
<th>26-Epi-bryostatin 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC-α</td>
<td>24 h</td>
<td>7 ± 1.0</td>
<td>1 ± 0.09</td>
<td>30 ± 5</td>
</tr>
<tr>
<td></td>
<td>4 days</td>
<td>2 ± 0.2</td>
<td>0.05 ± 0.007</td>
<td>37 ± 9</td>
</tr>
<tr>
<td>PKC-δ</td>
<td>24 h</td>
<td>4 ± 1.0</td>
<td>0.03 ± 0.008</td>
<td>4 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>4 days</td>
<td>0.8 ± 0.1</td>
<td>0.025 ± 0.01</td>
<td>1.6 ± 0.03</td>
</tr>
<tr>
<td>PKC-ε</td>
<td>24 h</td>
<td>N.A.*</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>4 days</td>
<td>N.A.*</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

* N.A., not applicable.
The dose-response curves for PMA are thus consistent with its effect on \([^3H]\)thymidine incorporation and proliferation. On the other hand, bryostatin 1 shows greater potency for the PKC isozymes \(\alpha, \delta, \) and \(\epsilon\) than it does for cell growth inhibition, suggesting that bryostatin 1 might inhibit cell growth via a pathway different from that of PKC.

**Effect of the PKC Inhibitor BIM-I on Growth Inhibition.** As a complementary approach, we tested the effect of a specific PKC inhibitor, BIM-I (26), on both PMA- and bryostatin 1-induced PKC activity and bryostatin 1-induced inhibition of \([^3H]\)thymidine incorporation. BIM-I completely inhibited PMA- and bryostatin 1-induced PKC enzymatic activity (Table 2). At the same doses tested, it inhibited the modest PMA-induced increase in \([^3H]\)thymidine incorporation, consistent with PKC's mediating this modest growth stimulation. In contrast, BIM-I did not block the inhibition of thymidine incorporation by bryostatin 1. Rather, it slightly enhanced the bryostatin-induced decrease in \([^3H]\)thymidine incorporation (Table 2). Interestingly, the combination of bryostatin 1 and BIM-I induced the same level of inhibition of \([^3H]\)thymidine incorporation as did 26-epi-bryostatin 1 alone (see below). The inhibitor results, like the dose-response curves, argue against a role for PKC in growth inhibition.

26-Epi-Bryostatin 1. As a third approach to assess the involvement of PKC in growth inhibition by bryostatin 1, we used a structural analogue of bryostatin 1, 26-epi-bryostatin 1, that had been shown to have a significantly lower affinity for PKC (21).

26-Epi-bryostatin 1 bound to recombinant PKC-\(\alpha\) with a 60-fold lower affinity than bryostatin 1. (The \(K_I\) value for bryostatin 1 was 0.54 \(\pm\) 0.04 \(nM\), and for 26-epi-bryostatin 1 it was 32.6 \(\pm\) 6.6 \(nM\).) This 60-fold reduced binding affinity in vitro was also reflected in the potency of 26-epi-bryostatin 1 to translocate and down-regulate the individual PKC isozymes.

PKC-\(\alpha\) was removed from the soluble fraction and translocated to the Triton X-100-soluble and -insoluble fractions with \(ED_{50}\) of 480 \(\pm\) 150 \(nM\) at 10 min, 26 \(\pm\) 7 \(nM\) at 24 h, and 20 \(\pm\) 2 \(nM\) at 4 days. It was down-regulated, with \(ED_{50}\) of 30 \(\pm\) 5 \(nM\) at 24 h and 37 \(\pm\) 9 \(nM\) at 4 days (Fig. 2B and Table 1).

PKC-\(\delta\) was removed from the soluble fraction, with \(ED_{50}\) of 39 \(\pm\) 5 \(nM\) at 10 min, 1.5 \(\pm\) 0.5 \(nM\) at 24 h, and 0.7 \(\pm\) 0.1 \(nM\) at 4 days (Fig. 4E and Table 1). At 24 h, the down-regulation of PKC-\(\delta\) by 26-epi-bryostatin 1, like that of bryostatin 1, followed a biphasic dose-response curve, with maximal down-regulation at 10–100 \(nM\) (Figs. 3B and 4F). As was the case for bryostatin 1, the down-regulation at these intermediate doses was not complete, between 50 and 70%. During the 4-day treatment schedule, 26-epi-bryostatin 1 showed the same biphasic dose-response curve for the down-regulation of this isozyme, with a more complete, about 90%, down-regulation (Fig. 4F).

PKC-\(\epsilon\) was removed from the soluble fraction, with \(ED_{50}\) of 26 \(\pm\) 6 \(nM\) at 10 min, 1.6 \(\pm\) 0.4 \(nM\) at 24 h, and 0.9 \(\pm\) 0.2 \(nM\) at 4 days

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**Table 2. Effect of BIM-I cotreatment on induction of PKC activity and \([^3H]\)thymidine incorporation in response to PMA or bryostatin 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BIM-I (1 (\mu M))</th>
<th>Fold increase of PKC activity</th>
<th>([^3H])Thymidine incorporation (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>0.54 (\pm) 0.03</td>
<td>91 (\pm) 2</td>
</tr>
<tr>
<td>PMA (10 (nM))</td>
<td>-</td>
<td>4.4 (\pm) 0.1</td>
<td>120 (\pm) 22</td>
</tr>
<tr>
<td>+</td>
<td>1.08 (\pm) 0.1</td>
<td>98 (\pm) 11</td>
<td></td>
</tr>
<tr>
<td>Bryostatin 1 (3 (\mu M))</td>
<td>-</td>
<td>3.02 (\pm) 0.3</td>
<td>43 (\pm) 2</td>
</tr>
<tr>
<td>Epi-bryostatin 1 (3 (\mu M))</td>
<td>+</td>
<td>1.07 (\pm) 0.04</td>
<td>25 (\pm) 5</td>
</tr>
<tr>
<td></td>
<td>N.D.</td>
<td>21 (\pm) 3</td>
<td></td>
</tr>
</tbody>
</table>

* a+, with BIM-I; –, without BIM-I.

b N.D., not determined.
Fig. 4. PMA-, bryostatin 1-, and 26-epi-bryostatin 1-induced changes in the levels of PKC-δ in the soluble (A, C, and E) and total (B, D, and F) fractions of B16/F10 melanoma cells. Melanoma cells were treated with the indicated doses of PMA, bryostatin 1, or 26-epi-bryostatin 1 for 10 min, 24 h, and 4 days. Samples for SDS-PAGE were prepared, and Western immunoblotting was performed as described in "Materials and Methods." The amount of enzyme was quantitated by densitometry and expressed as the percentage of the amount of isozyme present in the soluble fraction (A, C, and E) or in the total fraction (B, D, and F) in control cells, after normalization to the protein. Each point is the average of three to five independent experiments. Bars, SE.

At 24 h and on the 4-day treatment schedule, PKC-δ showed no significant down-regulation on 26-epi-bryostatin 1 treatment (Fig. 5B).

In the biochemical and cellular assays, 26-epi-bryostatin 1 showed a significantly reduced affinity for PKC. In good correlation with this, we found that 26-epi-bryostatin 1 is at least 10-fold less toxic than its natural counterpart, bryostatin 1.

To test the toxicity of bryostatin 1 and 26-epi-bryostatin 1, C57BL/6 mice were injected with either 500 or 1500 μg/kg 26-epi-bryostatin 1 or 150 or 500 μg/kg bryostatin 1. The animals were...
the indicated doses of bryostatin 1 for 10 min (A) and with the indicated doses of
bryostatin 1, PMA, and 26-epi-bryostatin 1. B16/F10 melanoma cells were treated with
obtained in two to four additional independent experiments.

Methods: “Equal amounts of proteins were loaded in each lane for the soluble (sol.; 10
Hg/lane), Triton X-100-soluble particulate (part.; 5 fig/lane), Triton X-100-insoluble
insol.

part.
sol.

PKC epsilon

Bryostatin 1

0.01 nM

0.1 nM

1 nM

10 nM

100 nM

1 μM

3 μM

control

10 min

sol.

part.

Insol.

B

PKC epsilon

Bryo

Epibryo

PMA

0.001 nM

0.1 nM

1 nM

10 nM

100 nM

1 μM

3 μM

control

24 hr

Fig. 5. Translocation of PKC-ε by bryostatin 1 and down-regulation of PKC-ε by
bryostatin 1, PMA, and 26-epi-bryostatin-1. B16/F10 melanoma cells were treated with
the indicated doses of bryostatin 1 for 10 min (A) and with the indicated doses of
bryostatin 1, PMA, and 26-epi-bryostatin 1 for 24 h (B). Samples for SDS-PAGE were
pre pared, and Western immunoblotting was performed as described in “Materials and
Methods.” Equal amounts of proteins were loaded in each lane for the soluble (sol.; 10
μg/lane), Triton X-100-soluble particulate (part.; 5 μg/lane), Triton X-100-insoluble
particulate (insol.; 10 μg/lane), and total (10 μg/lane) fractions. Identical results were
obtained in two to four additional independent experiments.

observed for 7 days. The higher dose of bryostatin 1 was lethal for all
animals tested by the third day after injection, whereas the lower dose of
bryostatin 1 and both of the 26-epi-bryostatin 1 doses caused only
relatively minor toxic effects. Because of the limited availability of
26-epi-bryostatin 1, we could not evaluate its possible toxicity at
higher doses.

Despite its significantly reduced affinity for PKC, 26-epi-bryostatin
1 displayed inhibitory potency similar to bryostatin 1 in the [3H]thy-
midine incorporation assay and showed only a 3-fold reduction in
potency in the chronic cell growth assay (Fig. 1, A and B). Furthermore,
the extent of inhibition both of [3H]thymidine incorporation and of
cell growth was greater than for bryostatin 1 in both assays (Fig. 1,
A and B). In the [3H]thymidine incorporation assay, the ED50 of
26-epi-bryostatin 1 was 600 ± 120 nM; the maximum inhibition of
[3H]thymidine incorporation was 80 ± 5% at the highest dose tested
(3 μM). Coapplication of BIM-I neither suppressed nor enhanced
the inhibition of [3H]thymidine incorporation by 26-epi-bryostatin 1 (Ta-
ble 2). On the chronic treatment schedule for cell growth, 26-epi-
bryostatin 1 showed an ED50 of 140 ± 30 nM, with a maximal
inhibition of 97 ± 3% at the highest dose tested (3 μM; Fig. 1, A and
B). During the 4-day treatment schedule, the number of cells in the
control group increased 80-100-fold. Therefore, these latter results
show that 3 μM 26-epi-bryostatin 1 induced almost complete growth
arrest. At the same time, the trypan blue exclusion test showed less
than 1% cell death, suggesting that 26-epi-bryostatin 1 at this dose is
a powerful cytostatic agent with no cytotoxic effect.

DISCUSSION

Our findings argue strongly that the cytostatic activity of the
bryostatins on the B16/F10 cells is mediated through a PKC-inde-
pendent mechanism: (a) for none of the PKC isozymes identified in
the B16/F10 cells, i.e., PKC-α, δ, and ε, is there a correlation between
the dose-response curves of bryostatin 1 for translocation or down-
regulation and for inhibition of proliferation; (b) The PKC inhibitor
BIM-I, which is active on all three PKC isozymes, α, δ, and ε, does
not block the inhibition of thymidine incorporation by bryostatin 1;
and (c) the bryostatin 1 analogue 26-epi-bryostatin 1 possesses po-
tency similar to bryostatin 1 as an inhibitor of cell growth. In contrast,
however, 26-epi-bryostatin 1 has 60-fold reduced potency for PKC
isozymes both as determined by phorbol ester binding in vitro and as
measured by PKC translocation and down-regulation in the intact
cells.

We have previously suggested that the protection of PKC-δ from
down-regulation by high doses of bryostatin 1 reflects interaction of
the bryostatin with a target distinct from that of the phorbol esters,
because the protection is noncompetitive with phorbol ester (10). This
protection of PKC-δ correlates with inhibition of increased c-Jun
expression in 3T3 fibroblasts (11) and with inhibition of phorbol ester
effects on cornified envelope formation and epidermal growth factor
binding in mouse keratinocytes (10). In contrast, the inhibition of
proliferation in the B16/F10 melanoma cells does not correlate with
the PKC-δ protection. Therefore, we assume that it represents a
distinct, PKC-independent mechanism.

The quantitative analysis of PKC isozyme translocation by bryo-
statin 1 and PMA in the B16/F10 melanoma cells shows marked
parallels with that observed previously in 3T3 fibroblasts (11) and
mouse keratinocytes (10). Bryostatin 1 causes slow translocation of
PKC-α and PKC-ε relative to PMA, whereas PKC-δ is translocated
slowly by both. In addition, bryostatin 1 is more potent for translo-
cating the novel isozymes PKC-δ and -ε compared with PKC-α. There
are also several noteworthy differences. First, PKC-α is dramatically
more sensitive to PMA in the B16/F10 melanoma cells than was
observed previously in keratinocytes (ED50 of 1 nM at 10 min com-
pared with 160 nM at 5 min or 65 nM at 20 min in the keratinocytes).
This difference emphasizes the crucial role that cellular context plays
in determining PKC isozyme structure-activity relationships. Second,
whereas the protection of PKC-δ in either NIH3T3 cells or mouse
keratinocytes was partial, corresponding approximately to the level of
PKC-δ present in the membrane fraction of unstimulated cells, in
the B16/F10 melanoma cells the level of PKC-δ in the presence of high
bryostatin 1 concentrations was similar to that in controls.

26-Epi-bryostatin 1 was quantitatively less potent than bryosta-
1 but behaved qualitatively in a generally similar fashion in its
actions on PKC. We were not able to distinguish whether the weak
activity observed by 26-epi-bryostatin 1 reflected minor (1–2)%
contamination by the natural epimer or was indeed intrinsic to
26-epi-bryostatin 1.

Our findings have important implications for the use of the bryo-
statins as cancer chemotherapeutic agents. Hornung et al. (2) reported
a close correlation between the potency of bryostatin 1 to suppress the
growth of tumor cell lines in tissue culture and in mice injected with
the same cell lines. We report here that 26-epi-bryostatin 1 was
similarly potent as an inhibitor of B16/F10 melanoma cell growth. We
would expect that the primary in vivo toxicity of bryostatin 1 would
reflect its interaction with PKC at inappropriate sites within the
animal. Consistent with this concept, 26-epi-bryostatin 1, modified in
a crucial element of the PKC pharmacophore, was less toxic in mice. Additional analysis of it and related C26-modified congeners as second-generation bryostatin derivatives for cancer chemotherapy may be of considerable interest.

It is important that our results not be prematurely overgeneralized, however. Bryostatin clearly has many actions related to its activation or antagonism of PKC. This activity on PKC may mediate its chemotherapeutic activity for some range of tumors, whether directly on the tumor cells or indirectly, e.g., by stimulation of the release of tumor necrosis factor (5). Likewise, for the current system, it will be important to confirm that the in vitro activity of 26-epi-bryostatin translates into an improved therapeutic index in the B16/F10-implanted tumor model.

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The Bryostatins Inhibit Growth of B16/F10 Melanoma Cells \textit{in Vitro} through a Protein Kinase C-independent Mechanism: Dissociation of Activities Using 26-Epi-Bryostatin 1


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