The in Vivo Effect of Bryostatin-1 on Paclitaxel-induced Tumor Growth, Mitotic Entry, and Blood Flow

Jason A. Koutcher, Monica Motwani, Kristen L. Zakian, Xiao-Kui Li, Cornelia Matei, Jonathan P. Dyke, Douglas Ballon, Hyok He Yoo, and Gary K. Schwartz

ABSTRACT

Pretreatment of tumor cells with the protein kinase C (PKC) inhibitor bryostatin-1 enhances the cytotoxicity of most chemotherapeutic agents. However, in the case of paclitaxel, this effect has been shown in vitro to be best achieved when bryostatin-1 follows (rather than precedes) paclitaxel treatment. With combination trials of bryostatin-1 and paclitaxel planned for clinical trials and with only in vitro data available regarding drug sequence, we elected to undertake an in vivo study evaluating the effect of sequential bryostatin-1 and paclitaxel in a tumor-bearing mouse model and to correlate this effect to cell cycle events, tumor metabolism, and tumor blood flow. At the maximum tolerated i.p. dose, bryostatin-1 at 80 μg/kg resulted in a small but significant increase in tumor doubling time (4.2 ± 0.3 days) compared with control tumors (3.0 ± 0.3 days; P < 0.01). Mice treated with i.v. paclitaxel, administered at a dose of 12 mg/kg every 12 h for three doses, weekly for 3 weeks, had a tumor doubling time of 23.4 ± 1.7 days. Mice pretreated with i.p. bryostatin-1 (80 μg/kg) followed 12 h later by i.v. paclitaxel (12 mg/kg every 12h for three doses) weekly for 3 weeks had a tumor doubling time of 9.7 ± 1.1 days. This was significantly less (P < .001) than paclitaxel alone, which indicated an inhibitory effect by bryostatin-1 on paclitaxel therapy. In comparison, tumor-bearing mice that were treated with the same dose but with the sequence of paclitaxel followed by bryostatin-1 had a tumor doubling time of 29.6 ± 0.6 days. This was significantly greater than the tumor doubling times for any condition tested (P < 0.01), demonstrating the sequence dependence of this combination. The efficacy of paclitaxel is dependent on mitotic entry, a step that requires activation of p34cdc2 kinase activity. Treatment with paclitaxel in vivo increased p34cdc2 kinase activity in the mouse mammary tumors, whereas administration of bryostatin-1 before paclitaxel prevented the p34cdc2 kinase activation by paclitaxel. This was further evaluated in vitro by flow cytometry in MKN-74 human gastric cancer cells. As determined by MPM-2 labeling, which identifies cells in mitosis, pretreatment with bryostatin-1 prevented paclitaxel-treated cells from entering mitosis. Bryostatin-1 has been reported to induce changes in muscle metabolism and to decrease muscle blood flow. These events could impact on the interaction of bryostatin-1 with paclitaxel. Using proton-decoupled phosphorus nuclear magnetic resonance (31P-NMR) spectroscopy in vivo, bryostatin-1 at 80 μg/kg induced a decrease in both intratumoral pH and high-energy phosphates. In vivo perfusion studies, using dynamic enhanced NMR imaging with gadolinium diethylenetriamine pentaacetic acid, also demonstrated decreased tumor blood flow. These studies suggest that the inhibition of tumor response to paclitaxel by bryostatin-1 is multifactorial and includes such diverse factors as inhibition of cell entry into mitosis, a decrease in pH and energy metabolism, and a decrease in tumor blood flow. These results indicate that, as this combination enters Phase I clinical trials, the sequence of paclitaxel followed by bryostatin-1 will be critical in the clinical trial design.

INTRODUCTION

Bryostatin-1 is a macrocyclic lactone obtained from the marine bryozoan, Bugilla Neritina. In vitro single-agent activity has been demonstrated against B16 melanoma, M5076 reticulum sarcoma, and L10A B-cell lymphoma (1, 2). Activity in vitro has also been observed in human A549 lung and MCF-7 breast cancer cells (3). Although the exact mechanism of action of bryostatin-1 is unknown, it has been shown in vitro to activate PKC3 activity with short-term exposure, by inducing translocation of the protein from its cytoplasmic to its membrane-associated active fraction. Conversely, prolonged exposure results in membrane depletion of PKC and results in inactivation of the PKC activity (4). Previous studies (5–10) have suggested that PKC inhibitors may have antineoplastic activity by mechanisms...
other than PKC inhibition. For example, the inhibition of cell growth by bryostatin-1 has also been correlated with the induction of p21 protein expression and the inhibition of CDK activity (5). In clinical trials, bryostatin-1 has also been associated with the release of tumor necrosis factor, the stimulation of interleukin 2, and an increase in interleukin 6 (7, 8). It has also been shown in muscle to induce changes in energy metabolism, decrease proton efflux, and reduce muscle blood flow (9, 10).

Bryostatin-1 has completed Phase I clinical trials, and different schedules of administration have been tested (11–13). Doses of bryostatin-1 above 65 μg/m² were associated with significant myalgias (12), the etiology of which remain unclear, although blood-flow inhibition was postulated as a likely candidate (9, 10). A bryostatin-1 dose of 25–50 μg/m² seems to be well tolerated and can be administered on a schedule of 3 out of every 4 weeks without dose-limiting toxicity. However, despite extensive Phase 1 testing, the results with single-agent bryostatin-1 have been discouraging. Limited responses have been observed in patients with ovarian carcinoma and lymphoma (11). The ultimate success of this agent may depend on new directions in its development.

Several reports indicate that inhibitors of PKC can significantly enhance the activity of chemotherapeutic agents. Bryostatin-1 has been shown to enhance the induction of apoptosis by Ara-C in HL-60 cells (14). The effect was best achieved when the cells were first treated with bryostatin-1 and then, 24 h later, treated with Ara-C. The enhancement of apoptosis, as determined by DNA fragmentation under these conditions, correlated with the enhanced inhibition of tumor growth as determined by clonogenic assays. Similar results have been reported with bryostatin-1 on the vincristine-treated WSU-DLC2 diffuse large-cell lymphoma cell line cells (15, 16) and on cisplatin-treated ovarian cancer cells (17). As in the case with Ara-C, this result was best achieved when bryostatin-1 treatment preceded that of either vincristine or cisplatin.

The sequential nature of this therapeutic approach in enhancing the effectiveness of a chemotherapeutic agent would clearly suggest that bryostatin-1 therapy should precede the cytotoxic agent under investigation. However this may not be the case for all drugs. Bryostatin-1 treatment in vitro has been reported to enhance the cytotoxicity of paclitaxel in human U937 leukemic cells only when bryostatin-1 followed paclitaxel treatment (18). This may be related to specific interactions between bryostatin-1 and paclitaxel-treated cells that are independent of bryostatin’s effect on PKC. In vitro studies with another PKC inhibitor, flavopiridol, have indicated that pretreatment of gastric and breast cancer cells with flavopiridol before paclitaxel will antagonize, rather than synergize, the paclitaxel effect. This antagonism by flavopiridol seems related to the inhibition by flavopiridol on paclitaxel-induced p34cdc2 kinase activity (19). The activation of cyclin B1-associated p34cdc2 kinase is critical for the effect of single-agent paclitaxel. Because bryostatin-1 is also a CDK inhibitor, we hypothesized that the antagonism of bryostatin-1 on paclitaxel-treated cells may also be related to bryostatin-1’s inhibition of paclitaxel-induced p34cdc2 kinase activity.

With combination trials of bryostatin-1 and paclitaxel planned for clinical trials and with only in vitro data available regarding drug sequence, we elected to undertake a study evaluating the effect of sequential bryostatin-1 and paclitaxel in a tumor-bearing mouse model. Toward this end, we have treated mice bearing a mouse mammary carcinoma with bryostatin-1 and paclitaxel alone or in sequential combinations (i.e., bryostatin-1 followed by paclitaxel or paclitaxel followed by bryostatin-1) and followed them for tumor growth. We selected a schedule of 3 out of 4 weeks because this has been shown to be safe and well tolerated in the clinical setting. We have also analyzed these tumors for p34cdc2 kinase activation, as well as for cyclin B1 protein expression, to determine the effects of these drugs on cell cycle events associated with mitotic arrest induced by paclitaxel. In view of the reported effects of bryostatin-1 on muscle metabolism with associated changes in ATP and a decrease in blood flow, we also elected to use MR spectroscopy (NMR) to serially measure in vivo the effects of bryostatin-1 on intratumoral energy, pH, and blood flow.

MATERIALS AND METHODS

In Vivo Tumor Model. MCA tumors were removed aseptically from tumor-bearing animals by previously described techniques (20). Briefly, a single-cell suspension was prepared by teasing and abrasion against stainless steel mesh immersed in iced MEM (Earle’s balanced salt solution) containing 2% heparin. Cell suspensions were further disrupted by aspiration through an 18-gauge needle, and the final suspension was agitated constantly by a magnetic spin bar. A tumor inoculum of 0.025–0.04 ml (approximately 10⁵ cells) was injected s.c. into the dorsum of the foot of male C3H/He mice (Jackson Laboratory, Bar Harbor, Me) with a 26-gauge needle. Greater than 75% of the MCA tumors were viable based on trypan blue exclusion. Tumor volume was estimated from the formula \( V = \pi d_1 \times d_2 \times d_3 \), where \( V \) = volume and \( d_1, d_2, \) and \( d_3 \) were the three orthogonal diameters. Different cohorts of mice were used for NMR and tumor-growth-delay studies. Mice were fed ad libitum until just before NMR and radiation studies.

Cell Culture. The human gastric cancer cell line MKN-74 was used for in vitro studies because the MCA tumor cannot be grown in vitro. The MKN-74 cell was graciously supplied by Dr. E. Tahara (Hiroshima University, Hiroshima, Japan). Cells were maintained in Eagle’s MEMs supplemented with 20% heat-inactivated normal calf serum (Intergen), penicillin, and streptomycin at 37°C in 5% carbon dioxide. The cultures were tested as Mycoplasma-free.

Tumor Response and Toxicity Studies. Mice with tumors approximately 100 mm³ in size were used for both TDT and LD10 (dose that kills 10% of mice) studies. The LD10 studies were done with a single administration of bryostatin-1 i.p. at the doses noted (Table 1). Toxicity was evaluated at day 7 because the tumor had grown to ~700 mm³ and, therefore, the mice were killed. For evaluation of tumor response for the combined paclitaxel and bryostatin-1 studies, mice were treated with bryostatin-1 (supplied by Bristol-Meyers Squibb, Princeton, NJ) i.p. or paclitaxel (Bristol-Meyers Squibb) i.v. Each cycle of paclitaxel was administered at a dose of 12 mg/kg i.v. for three doses, each separated by 12 h. Mice received: (a) saline (\( n = 10 \)); (b) bryostatin-1 (80 μg/kg; \( n = 10 \)); (c) paclitaxel (12 mg/kg every 12 h for three doses; \( n = 10 \)); (d) bryostatin-1 (80 μg/kg) followed 12 h later by the first of three doses of pacli-
Interaction of Paclitaxel and Bryostatin-1

Paclitaxel (each dose of paclitaxel separated by 12 h; n = 14); or (e) paclitaxel (12 mg/kg for three doses, each separated by 12 h, followed by bryostatin-1 (80 μg/kg; 12 h after the last dose of paclitaxel; n = 14). The 12-h time interval of administering paclitaxel after the last dose of bryostatin-1 was selected because this was the point at which intratumoral energy and high-energy phosphates were the lowest after bryostatin-1 therapy (see “Results” section, “Effect of Bryostatin-1 on Intratumoral Energy and pH”). For TDT studies of the combinations of paclitaxel and bryostatin-1, the mice received three cycles of each treatment, separated by each week.

p34<sup>Here</sup> Kinase Activity Assay. For these assays, tumors were obtained from all of the sets of animals 12 h after completing day 2 of therapy. Tumors were then homogenized and lysed with buffer containing 50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM DTT, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 10 mM β-glycerophosphate, 0.1 mM Na<sub>2</sub>VO<sub>4</sub>, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The cells were further disrupted by passing through a 21-gauge syringe 10 times and lysates were clarified by centrifugation (10 min at 10,000 × g). Soluble protein (200 μg) was incubated with 1 μg of anticytokerin B1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C for 2 h. Immune complexes were then precipitated with 40 μl of immobilized protein A (RepliGen, Weedham, MA) overnight at 4°C, washed three times with lysis buffer and twice with kinase assay buffer (50 mM HEPES-KOH (pH 7.5), 10 mM MgCl<sub>2</sub> and β-glycerophosphate, 1 mM DTT, 2.5 mM EGTA, 0.1 mM Na<sub>2</sub>VO<sub>4</sub>, 1 μM NaF). The kinase assay was done by combining the washed protein beads with 20 μl of kinase buffer plus 10 μCi of [γ<sup>32</sup>P]ATP, 15 μM ATP, and 50 μg/ml Histone H1 (Boehringer Mannheim, Germany). The reaction was allowed to proceed for 30 min at 30°C and was terminated by adding 10 μl of Laemmli sample buffer and boiling for 5 min. Products were resolved by 10% SDS-PAGE. The incorporated radioactivity was determined by Betascope 603 blot analyzer (Betagen Corp., Waltham, MA).

Immunoblot Analysis. Protein lysates, prepared for kinase assays, were used for immunoblotting. Soluble protein (50 μg) was resolved by 10% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). The equal loading of proteins was confirmed by Amido Black staining. The membranes were probed with mouse monoclonal cyclin B<sub>1</sub> (kindly provided by Dr. Tim Hunt, Imperial Cancer Research Fund Clare Hall Laboratories, United Kingdom). The membrane was treated with a secondary sheep antimouse-horseradish peroxidase antibody for 1 h at room temperature. Detection was done by ECL chemiluminescence reagents (DuPont NEN Life Science Products, Boston, MA) according to the manufacturer’s protocol. The levels of expressions were quantified using a densitometric scanning system.

MPM-2/Propidium Iodide Bivariate Flow Cytometry. MKN-74 cells (1.4 × 10<sup>7</sup>/100-mm dish) were cultured for 48 h and treated with paclitaxel (100 nm) as a single agent for 18 h (T<sub>18</sub>) or sequentially with 1 μM bryostatin-1 for 24 h followed by paclitaxel for 18 h (Bryo<sub>24</sub>→T<sub>18</sub>). The cells were harvested after paclitaxel therapy by trypsinization, pooled with floating cells, and fixed with 1% paraformaldehyde and 70% ethanol. After washing with PBS containing 0.05% Tween 20 and 1% fetal bovine serum, cells were labeled with MPM-2 antibody (final concentration of 6 μg MPM-2 antibody/ml; Upstate Biotechnology, Lake Placid, NY) for 1 h at 4°C. Cells were washed twice with PBS and incubated with goat antimouse-FITC (Boehringer Mannheim, Mannheim, Germany) for 1 h at room temperature in the dark. After washing twice with PBS, cells were resuspended in 5 μl/ml propidium iodide containing 50 μg/ml RNase A. Samples were analyzed on a Becton Dickinson FACScan, and data were analyzed using CellQuest software. The MPM-2 positive cells (mitotic cells) will show increased green fluorescence, thus shifting above the baseline of the dot plot.

NMR. Proton-decoupled <sup>31</sup>P-NMR spectra were obtained from MCA tumors on the dorsal aspect of the foot of C3H mice at 81.03 MHz on a Bruker CSI 4.7T 33-cm horizontal bore magnet (Fremont, CA). The unanesthetized mouse was suspended in an acrylic former with the leg immobilized and extended horizontally so that the tumor-bearing foot was surrounded by a 13-mm internal diameter four-turn teflon-coated solenoidal coil tuned to 81 MHz. Experimental parameters included a spectral width of 10,000 Hz, delay of 2 s, 60° flip angle, 4096 data points and 2048 free induction decays. The WALTZ-16 (Wonderful Alternating Phase technique for Zero Residual Splitting) routine was used to decouple during the acquisition. The free induction decays were zerofilled once, a double exponential multiplication filter of 6 was applied, and the transformed spectra were manually baseline-corrected. A single 13-mm internal diameter four-turn teflon-coated coil was used. Field homogeneity was improved, and temperature was maintained by immersing the tumor-bearing foot in a water bath at 37°C. Peak areas were estimated from the spectra by fitting the spectra to a series of Lorentzian peaks while varying the peak position, height, and line-width to obtain the best fit using software available on the spectrometer (SPANTOOL). A decrease in the ratio of the PCr to Pi peak areas (PCr:Pi) is indicative of decrease energy status. Tumor pH is estimated from the chemical shift of the Pi resonance relative to PCr. Tumors were studied by NMR after attaining a volume of ~250 mm<sup>3</sup>. Mice were treated with 160 μg/kg (n = 4), 120 μg/kg (n = 8), 80 μg/kg (n = 8), and 60 μg/kg (n = 8).

Tumor Perfusion. To analyze tumor perfusion, animals were studied using rapid MR imaging with Gd-DTPA contrast (Magnevist, Berlex Laboratories, Wayne, NJ). All of the studies were performed on a 4.7 Tesla Bruker-CSI imager with a 33-cm horizontal bore. Perfusion was studied 12 h after treatment with either 80 or 120 μg/kg of bryostatin-1 (n = 7 per group). To avoid the possibility of the remaining Gd-DTPA influencing the results, it was decided that baseline and posttreatment gadolinium studies should not be performed on the same mouse, although subsequent control experiments did not note any effect on signal intensity 12 h after injection of Gd-DTPA. A separate group of control animals with tumors in the same size range were used to provide baseline data. Tumor volumes ranged from 200 to 250 mm<sup>3</sup> for the perfusion studies.

In all of the perfusion studies, a 24-gauge i.v. catheter was introduced into the tail vein, and a syringe containing Gd-DTPA (0.02 ml diluted to a volume of 0.3 ml) was attached to the catheter. The unanesthetized mouse was suspended in an acrylic former with the leg immobilized and extended horizontally so...
that the tumor-bearing foot was surrounded by a 2-turn, 1.5-cm-diameter solenoidal coil tuned to 200 MHz. Spin-lattice (T1)-weighted spin-echo scout images were acquired to localize the tumor and to prescribe the largest cross-sectional slice for dynamic imaging. A single 1.5-mm thick slice was imaged at high resolution [repeat interval (TR), 300 ms; echo time (TE), 10 ms; number of acquisitions (NA), 8; field of view (FOV), 40 mm; matrix, 256 × 128; voxel size, 0.156 × 0.3125 × 1.5 mm]. The same slice was then imaged dynamically using a spin-echo pulse sequence (TR, 100 ms; TE, 10 ms; NA, 2; 12.8 s per image; FOV, 40 mm; matrix, 64 × 64; voxel size, 0.62 × 0.62 × 1.5 mm). After 1 min, a bolus injection of Gd-DTPA was given, and imaging continued for 12.6 additional min.

After on-line reconstruction, data were exported to a Sun Ultra 1 workstation (Sun Microsystems, Mountain View, CA.) for analysis. In-house software was written to display and analyze the data using IDL 5.1 (Research Systems Inc., Boulder, CO.). For each tumor, the time course of signal intensity in three ROI was examined. These regions encompassed the center, the rim, or the entire tumor cross-section, respectively. The high resolution T1-weighted scout image was used for more accurate selection of ROI. Care was taken to avoid any contamination of the ROI due to the bones of the foot and normal adjacent tissue. For each ROI, the maximum slope and baseline intensity were determined. To search for the maximum slope, a five-point, 64-s, sliding window was applied to the first 3 min of the time-intensity curve (21). The window search method compensated for regional heterogeneity in the time point of initial uptake. The slope was determined by linear regression of the five time points within the window. The maximum slope within 2 min after injection of the contrast agent was found. The baseline value was calculated as the mean of the points prior to the maximum slope window. The maximum percent signal increase (ΔSI_max) per unit time in the ROI was calculated according to the equation given below:

\[
\% SI_{\text{max}} = \frac{\text{Maximum slope} \times 100}{SI_{\text{pre}}}
\]

where the SI_pre value was the baseline average signal intensity. This equation represents a slight modification of the technique reported by Erlemann et al. (22). The identical algorithm was also applied to each voxel to generate parametric images of maximum percent signal intensity increase per unit time.

Statistical Analysis. All of the in vitro experiments were repeated at least three times unless otherwise indicated. The statistical significance of the animal studies was determined by the two-sided Student’s t test from the mean ± SE.

RESULTS

Effect of Bryostatin-1 and Paclitaxel on Tumor Growth Delay. Table 1 shows the effect of different single doses of bryostatin-1 on tumor growth delay and survival. Bryostatin-1 as a single agent had modest activity in this tumor model. The maximum tolerated dose (LD10) for this tumor model was 80 µg/kg, for a single dose of bryostatin-1, which induced a TDT of 4.2 ± 0.3 days (n = 10), compared with 3.0 ± 0.3 days (n = 10) for untreated mice (t = 3.08; P < 0.01). Bryostatin-1 failed to show a dose response effect in the dose range of 50–80 µg/kg. The TDTs for mice treated with bryostatin-1 at both 50 and 60 µg/kg were also modestly greater than controls. A dose of 100 µg/kg induced a greater tumor growth delay for mice that survived the treatment. Nevertheless, this dose exceeded the acceptable mortality rate and was deemed toxic. Because 80 µg/kg of bryostatin-1 was the maximum tolerated dose in this tumor model, it was selected as the dose for the combination studies with paclitaxel.

The effect of treatment on tumor growth with the different drugs for three weekly cycles was evaluated. The effect of single agent bryostatin-1 could not be evaluated beyond 7–10 days because there was minimal tumor response and the tumors had grown to the point that the animals required sacrificing. The TDT after treatment with paclitaxel was 23.4 ± 1.7 days (n = 10). This is compatible with the drug’s known activity in breast cancer (23). With bryostatin-1 followed by paclitaxel (bryostatin-1→paclitaxel), the TDT was decreased to 9.7 ± 1.1 days (n = 14), which indicated an antagonistic effect by bryostatin-1 on the paclitaxel-treated tumors. For mice that received the reverse combination of paclitaxel followed by bryostatin-1, the TDT was prolonged to 29.6 ± 0.6 days (n = 14; paclitaxel→bryostatin-1), which suggested an additive effect when compared with paclitaxel alone. The differences between paclitaxel, paclitaxel→bryostatin-1, and bryostatin-1→paclitaxel were all significant (P < 0.01). These in vivo data indicate that pretreatment with bryostatin-1 before paclitaxel will antagonize the effect of paclitaxel and emphasize that the interactions between bryostatin-1 and paclitaxel are sequence-dependent.

Effect of Bryostatin-1 on Paclitaxel-induced p34^{cdk2} Kinase Activity. One possible reason for the decreased response to the sequential bryostatin-1 followed-by-paclitaxel combination may be related to a cell cycle effect of bryostatin-1 on

<table>
<thead>
<tr>
<th>Dose</th>
<th>% mortality (7 days)</th>
<th>TDT (days)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0 (0/5)</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>Bryostatin 50 µg/kg</td>
<td>10 (1/10)</td>
<td>4.1 ± 0.3 (P &lt; 0.05 compared with control)</td>
</tr>
<tr>
<td>Bryostatin 60 µg/kg</td>
<td>10 (1/10)</td>
<td>5.0 ± 0.7 (P &lt; 0.05 compared with control)</td>
</tr>
<tr>
<td>Bryostatin 80 µg/kg</td>
<td>10 (1/10)</td>
<td>4.2 ± 0.3 (P &lt; 0.01)</td>
</tr>
<tr>
<td>Bryostatin 100 µg/kg</td>
<td>40 (2/5)</td>
<td>6.9 ± 0.1* (P &lt; 0.01 compared with 80 µg/kg) (P &lt; 0.0 compared with control)</td>
</tr>
<tr>
<td>Bryostatin 120 µg/kg</td>
<td>100 (4/4)</td>
<td></td>
</tr>
<tr>
<td>Bryostatin 160 µg/kg</td>
<td>100 (5/5)</td>
<td></td>
</tr>
</tbody>
</table>

*Based on mice that survived ≥7 days.
Interaction of Paclitaxel and Bryostatin-1

For 18 h (T18), 56% of the cells stain positively for MPM-2, positively for MPM-2. However, with paclitaxel treatment alone in Table 2, in untreated cells (No drug) only 2% of the cells stain pear during mitosis (26). Two-dimensional flow cytometry was tibody recognizes epitopes shared by phosphoproteins that ap-

mitotic cells was determined by MPM-2 labeling. MPM-2 an-

anapy

MKN-74 cells with bryostatin-1 for 24 h before paclitaxel with the treatment of bryostatin-1 prevents the activation of p34cdc2 kinase, with the untreated controls. This would indicate that pretreat-

ment alone causes an accumulation of cells in G1.

**Effect of Bryostatin-1 on Intratumoral Energy and pH.** Because energy metabolism has been shown to be involved in apoptosis [although it remains controversial whether this repre-
sents a cause or effect (27)], and bryostatin-1 has been reported to change energy metabolism in muscle, we evaluated the effect of bryostatin-1 on intratumoral energy and pH by NMR. Fig. 2 shows a series of 31P-NMR spectra obtained before and after bryostatin-1 at multiple time points after 80 (Fig. 2A) and 60 µg/kg (Fig. 2B) of bryostatin-1. The spectra showed peaks arising from the phosphomonoesters [phosphoethanolamine (peaks A) and phosphocholine (peaks B), Pi (peaks C), phospho-

phidiesterases [glycerophosphoethanolamine (peaks D), and glyc-

erophosphocholine (peaks E)]. PCr (peaks F), and nucleoside

triphosphates [γNTP (peaks G), αNTP (peaks H), NAD(H) (peaks I), βNTP (peaks J)]. As expected, the peaks are well resolved, which facilitates quantitation. At 12 h after treatment with bryostatin-1, a reduction in the peak areas of PCr:Pi was noted. Fig. 3 shows the changes in PCr:Pi and pH noted after the treatment of eight tumor-bearing mice with bryostatin-1 at a dose of 80 µg/kg. A decrease in PCr:Pi at 12 h was statistically significant (P < 0.03). This indicates a loss of high-energy phosphate (PCr) and an increase in free Pi and is consistent with a decrease in intratumoral energy. This effect persisted up to 40 h after bryostatin-1 therapy. Depletion in energy at 12 h after 120 µg/kg (n = 4 animals; data not shown) was greater than after 80 µg/kg, although the differences did not attain signifi-
cance (P < 0.10). After treatment with 80 µg/kg of bryostatin-1, there is a maximum drop of 0.13 ± 0.02 pH units noted at 12 h posttreatment, which shows recovery by 48 h. It is noted that, after treatment with bryostatin-1, the Pi peak (peak C) shifts upfield, which is indicative of tumor acidosis, compared with the pretreatment pH. As shown in Fig. 2, the Pi peak was also often split into multiple resonances indicative of pH heteroge-

neity in the tumor.

Table 2  Effect of sequential bryostatin-1 and paclitaxel on mitotic arrest

<table>
<thead>
<tr>
<th>Conditions</th>
<th>G1 (%)</th>
<th>S phase (%)</th>
<th>G2 (%)</th>
<th>M (%)</th>
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<tr>
<td>No drug</td>
<td>34</td>
<td>47</td>
<td>17</td>
<td>2</td>
</tr>
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<td>3</td>
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<td>28</td>
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<td>23</td>
</tr>
<tr>
<td>T18 → Bryo24</td>
<td>9</td>
<td>18</td>
<td>69</td>
<td>4</td>
</tr>
</tbody>
</table>

Fig. 1 Effect of sequential bryostatin-1 and paclitaxel on cyclin B1 expression and p34cdc2 kinase activation. Mice bearing MCa tumors were treated with paclitaxel (T) or sequential bryostatin-1 followed by paclitaxel (Bryo→T) as described in “Materials and Methods.” Tumor extracts were prepared, and protein levels were analyzed by immuno-

blotting for cyclin B1. Using the Histone H1 kinase assay as described in “Materials and Methods,” the kinase activity of p34cdc2 kinase was examined in the tumor extracts under identical conditions. Results from a representative experiment are shown.

Effect of Bryostatin-1 on Mitotic Entry of Paclitaxel-treated Gastric Cancer Cells. Because bryostatin-1 prevents p34cdc2 kinase activation by paclitaxel, we evaluated the effect of bryostatin-1 on paclitaxel-induced mitotic entry. This is dif-

ficult to evaluate in vivo, and, therefore we elected to examine the effect of sequential bryostatin-1 followed by paclitaxel therapy in vitro in MKN-74 gastric cancer cells. The percentage of mitotic cells was determined by MPM-2 labeling. MPM-2 antitbody recognizes epitopes shared by phosphoproteins that appear during mitosis (26). Two-dimensional flow cytometry was used to quantitate the population of cells in M phase. As shown in Table 2, in untreated cells (No drug) only 2% of the cells stain positively for MPM-2. However, with paclitaxel treatment alone for 18 h (T18), 56% of the cells stain positively for MPM-2, consistent with mitotic entry. In contrast, pretreatment of MKN-74 cells with bryostatin-1 for 24 h before paclitaxel (Bryo24→T18) resulted in only 23% of the cells staining positively for MPM-2. This would suggest that pretreatment of cells with bryostatin-1 before paclitaxel prevented the cells from entering into mitosis. Prevention of entry into mitosis by pre-
treatment with bryostatin-1 also antagonizes the effect of palci-
taxel in these cells (data not shown). With the reverse sequence of T18→Bryo24, the cells that had entered the M phase with paclitaxel alone (56%) have clearly exited M (4%) after 24 h of additional bryostatin-1 therapy. Interestingly, bryostatin-1 treat-

ment alone causes an accumulation of cells in G1.

Pacoxitaxel-treated tumor cells. The effect of paclitaxel is de-

pendent on metaphase arrest, which requires the activation of p34cdc2 kinase activity. p34cdc2 kinase activity is induced by its association with cyclin B1 and dephosphorylation of cdc2 at Thr (14) and Tyr (15). Cyclin B1 is induced in G2 and continues to accumulate until metaphase (24, 25). We assayed cyclin B1-associated p34cdc2 kinase activity in the MCa tumor by histone H1 phosphorylation immediately after completing week 1 of therapy. As shown in Fig. 1, treatment with paclitaxel (T) alone resulted in a 20% increase in p34cdc2 kinase activity when compared with the untreated controls (ND). This was associated with a 60% increase in cyclin B1 protein expression. However, with the treatment of bryostatin-1 followed by paclitaxel (Bryo→T), p34cdc2 kinase activity was decreased 10-fold, and cyclin B1 protein expression was decreased 100-fold, compared with the untreated controls. This would indicate that pretreat-

ment with bryostatin-1 prevents the activation of p34cdc2 kinase, a cell cycle event that is critical for paclitaxel’s anticancer effect.

Effect of Bryostatin-1 on Intratumoral Energy and pH. Because energy metabolism has been shown to be involved in apoptosis [although it remains controversial whether this repre-
sents a cause or effect (27)], and bryostatin-1 has been reported to change energy metabolism in muscle, we evaluated the effect of bryostatin-1 on intratumoral energy and pH by NMR. Fig. 2 A

shows a series of 31P-NMR spectra obtained before and after bryostatin-1 at multiple time points after 80 (Fig. 2A) and 60 µg/kg (Fig. 2B) of bryostatin-1. The spectra showed peaks arising from the phosphomonoesters [phosphoethanolamine (peaks A) and phosphocholine (peaks B), Pi (peaks C), phospho-

phidiesterases [glycerophosphoethanolamine (peaks D), and glyc-
erophosphocholine (peaks E)]. PCr (peaks F), and nucleoside

triphosphates [γNTP (peaks G), αNTP (peaks H), NAD(H) (peaks I), βNTP (peaks J)]. As expected, the peaks are well resolved, which facilitates quantitation. At 12 h after treatment with bryostatin-1, a reduction in the peak areas of PCr:Pi was noted. Fig. 3 shows the changes in PCr:Pi and pH noted after the treatment of eight tumor-bearing mice with bryostatin-1 at a dose of 80 µg/kg. A decrease in PCr:Pi at 12 h was statistically significant (P < 0.03). This indicates a loss of high-energy phosphate (PCr) and an increase in free Pi and is consistent with a decrease in intratumoral energy. This effect persisted up to 40 h after bryostatin-1 therapy. Depletion in energy at 12 h after 120 µg/kg (n = 4 animals; data not shown) was greater than after 80 µg/kg, although the differences did not attain signifi-
cance (P < 0.10). After treatment with 80 µg/kg of bryostatin-1, there is a maximum drop of 0.13 ± 0.02 pH units noted at 12 h posttreatment, which shows recovery by 48 h. It is noted that, after treatment with bryostatin-1, the Pi peak (peak C) shifts upfield, which is indicative of tumor acidosis, compared with the pretreatment pH. As shown in Fig. 2, the Pi peak was also often split into multiple resonances indicative of pH heteroge-

neity in the tumor.
Effect of Bryostatin-1 on Tumor Blood Flow. Another possible reason for decreased response (when mice were treated initially with bryostatin-1 followed by paclitaxel) was decreased drug delivery or changes in the tumor microenvironment secondary to decreased tumor blood flow. Previous studies (28, 29) have indicated that the initial rate of uptake of Gd-DTPA is related to perfusion. Therefore, to analyze tumor perfusion, animals were studied using rapid MR imaging with Gd-DTPA 12 h after treatment with 80 μg/kg of bryostatin-1 (n = 7). The average signal-intensity-enhancement time-course curve for control animals, and for mice treated with 80 μg/kg of bryostatin-1, is shown in Fig. 4. There is a slower rate of rise of the signal in both the middle part of the tumor and particularly the rim after bryostatin-1. Similarly, there is a decrease in the maximum signal intensity attained. In Table 3, the maximum percent signal enhancement and maximum slope are shown.

Fig. 2 A. 31P-NMR spectra obtained before and after 80 μg/kg bryostatin-1. Peaks include A, phosphoethanolamine; B, PC; C, Pi; D, glycerophosphoethanolamine; E, glycerophosphocholine; F, PCr; G, γNTP; H, αNTP; I, NAD(H); and J, βNTP. The Pi peak shifts upfield (to the right) after treatment, which is indicative of a decrease in pH. B, 31P-NMR spectra obtained before and after 60 μg/kg bryostatin-1. There is a decrease in PCr peak relative to the Pi peak after treatment with bryostatin-1.
Interaction of Paclitaxel and Bryostatin-1

The anticipated effect of this on the cell cycle of these in vivo systems, and perfusion. This is the first report of signal intensity versus time was seen at bryostatin-1 doses of 80 µg/kg in the rim area (P < 0.005), which suggests a decrease in tumor perfusion in the rim of the tumor. Changes in the central region of the tumor were not significantly different after bryostatin-1 (P = 0.20). Averaging the result over the whole tumor volume indicated that bryostatin-1 did induce a change in the maximum slope (P < 0.03). Control studies indicated that the effect of paclitaxel on perfusion in the rim of the tumor approached, but did not attain, statistical significance (P < 0.08).

**DISCUSSION**

We have investigated the effect of bryostatin-1 in combination with paclitaxel on tumor growth and on paclitaxel-induced p34cdc2 kinase activity, mitotic entry, tumor metabolism, and perfusion. This is the first in vivo data to indicate that pretreatment with bryostatin-1 decreases the efficacy of paclitaxel, whereas the reverse sequence (paclitaxel→bryostatin-1) shows a statistically significant increase in efficacy compared with single-agent paclitaxel. Therefore, in contrast to other drug combinations, pretreatment with bryostatin-1 will antagonize rather than enhance the paclitaxel effect. Our results indicate that, in mouse mammary tumors, bryostatin-1 blocks the activation of p34cdc2 kinase activity in association with a decrease in the protein expression of the p34cdc2 kinase activator, cyclin B1. The anticipated effect of this on the cell cycle of these mouse mammary tumors is to decrease the percentage of cells entering mitosis when treated with paclitaxel. MCA tumors grow only in vivo and are not amenable to in vitro analysis by flow cytometry. Using the MKN-74 gastric cancer cell line, we were able to show by flow cytometry that the treatment of tumor cells with bryostatin-1 before paclitaxel decreases mitotic entry. In addition, in vivo NMR studies indicate that bryostatin-1 induces a decrease in intratumoral energy, pH, and blood flow that may adversely affect the efficacy of subsequent paclitaxel therapy.

To investigate the reason for the decreased efficacy of paclitaxel after treatment with bryostatin-1, we first investigated the effect of bryostatin-1 on mitotic events that pertain to paclitaxel. Paclitaxel is a mitotic spindle inhibitor and induces mitotic block in a variety of cell lines (30). The critical events associated with paclitaxel-induced apoptosis are not known; however, it has been shown that mitotic block induces apoptosis by stabilizing microtubule dynamics rather than by alteration of the microtubule mass (31). The importance of mitotic block in inducing apoptosis in response to paclitaxel has also been shown by various groups using antisense of cyclin B1 to abrogate the p34cdc2 kinase activity (32). The prevention of mitotic block also prevents the cell death. Therefore, we elected to explore whether there were cell cycle events relative to p34cdc2 kinase activity that could explain the lack of tumor response relative to the sequential bryostatin-1 followed by paclitaxel therapy. Analysis of the tumor tissues shows that bryostatin-1 inhibits p34cdc2 kinase activity in association with a decrease in the expression of cyclin B1 protein, the p34cdc2 kinase activator.

Further examination of this event in an in vitro system also indicated that bryostatin-1 pretreatment prevents entry of tumor cells into mitosis by paclitaxel. Because mitotic entry is critical for the paclitaxel effect, these results indicate that the timing of bryostatin-1 relative to paclitaxel will inhibit the activation of p34cdc2 kinase activity by paclitaxel, inhibit mitotic entry, and antagonize the paclitaxel effect. Although the latter study was done in a different tumor model, it still supports the hypothesis that pretreatment of tumor cells with bryostatin-1 will block the effect of paclitaxel.

These studies do not exclude the importance of other cell cycle events in this process. For example, Asiedu et al. (5) has reported in vitro that bryostatin-1 induces p21 in U937 leukemic cells and inhibits CDK2 activity. These events are capable of inducing a G1 arrest, which could also prevent paclitaxel treated cells from entering the M phase of the cell cycle, thus antagonizing the paclitaxel effect. This may, in fact, explain the increase in the G1 population observed in the MKN-74 cells after treatment with single-agent bryostatin-1. Wang et al. (18) has reported in vitro an increase in the induction of apoptosis of U937 human leukemic cells when bryostatin-1 was given after, not before, paclitaxel. They attribute this effect, in part, to an increase in the amount of free Bax, a proapoptotic protein that is not detected in our cells. However, we could not detect changes in the bax:bcl-2 ratio, nor could we detect an increase in p21 in our tumor cells (data not shown). Nevertheless, with the bryostatin-1-paclitaxel combination, there seems to be a sequential dependency that depends on an ordering of events relative to cell cycle effect.

Hickman et al. (9) and Thompson et al. (10), in previous studies, demonstrated that bryostatin-1 induces changes in muscle metabolism. Specifically, they noted a significant increase in the phosphodiester:ATP ratio at 48 h after bryostatin-1 treatment, which suggests a decrease in high-energy phosphates. This could result in antagonism of the subsequent paclitaxel effect. Hydrolysis of high-energy phosphates, especially GTP, has been shown to play an important role in paclitaxel-induced microtubule assembly (33, 34). Thus, the decrease in high-energy phosphates induced by bryostatin-1 in vivo could result in impaired tubulin polymerization by subsequent paclitaxel therapy.

![Graph](image-url)
Hickman et al. (9) also noted a decrease in the proton efflux rate at 4 h after bryostatin-1, which indicated a decrease in muscle blood flow. In three of four patients studied with near-IR spectroscopy, they found a reduced rate of postexercise reoxygenation that was consistent with reduced blood flow. This was associated with impaired mitochondrial energy production.

Fig. 4  Percent change in tumor signal intensity after a dose of Gd-DTPA in control tumor-bearing mice or 12 h after the tumor-bearing mice were treated with bryostatin-1. A, rim of tumor; B, central core of tumor; C, whole tumor. There were modest differences in the center and rim of the tumor between control and tumor-bearing mice treated with bryostatin-1, which approached significance. The change in maximum enhancement over the whole tumor was significant ($P = 0.05$).
which was consistent with either vasoconstriction or direct mitochondrial toxicity. Additional studies were carried out attempting to reverse the myalgias, induced by bryostatin-1, by treatment with nifedipine. They concluded that bryostatin-1 likely did induce vasoconstriction but that it was not the cause of the bryostatin-1-induced myalgias.

The mouse mammary carcinoma perfusion studies, although not proving cause, indicated that tumor perfusion decreased after bryostatin-1. This in itself could explain the decrease in pH and PCr:Pi. In nearly all of the tumor-bearing animals treated with 80 μg/kg of bryostatin-1 (seven of eight), splitting of the Pi peak (or enhanced splitting compared with pretreatment) was detected within the first 24 h of treatment, which indicated a greater heterogeneity of tumor pH favoring acidosis. This suggested that the effect induced by bryostatin-1 on the tumor was not uniform. This is also more suggestive of an effect on blood flow, as opposed to an inhibition of a biochemical process, because tumor blood flow is typically heterogeneous. This heterogeneity is further confirmed by the perfusion studies, which showed variation over the image in the parametric plots of changes in signal over time.

This decrease in tumor blood flow by bryostatin-1 may explain the slight increase in TDT with bryostatin-1 as a single agent. This also may result in a decrease in drug delivery of subsequent paclitaxel therapy to the tumor site. Additional studies with a NMR-visible drug (5-fluorouracil) are under way to determine whether bryostatin-1 decreases drug delivery to the tumor; this would corroborate the hypothesis that the inhibition of cell kill after pretreatment with bryostatin-1 is in part due to decreases in perfusion.

Treatment with bryostatin-1 induced a decrease in tumor pH. Previous studies (35–37) have demonstrated that small decreases in pH can increase the induction of apoptosis. This would suggest that pretreatment with bryostatin-1 might enhance, rather than antagonize, the effect of paclitaxel. However, this was not the case in our model system. One possibility for this is that the cytotoxicity of paclitaxel is decreased at a reduced pH (38). A decrease in pH has also been associated with increased accumulation of cells in the G1 phase of the cell cycle, in which they are more resistant to the effect of the drug (38).

The dynamic ordering of events seems to be of critical importance, as the combination of paclitaxel and bryostatin-1 enters clinical trials. Alterations in particular events associated with the interactions between these two drugs, especially the effects on energy metabolism and blood flow, could only have been detected in an in vivo system. On the basis of these laboratory studies, we have launched a Phase I clinical trial of weekly sequential paclitaxel and bryostatin-1 (39). On the basis of our preclinical models, patients are treated for at least 3 weeks with a fixed dose of paclitaxel on day 1 followed by bryostatin-1 at escalating doses in each cohort on day 2 of the weekly therapy. In this clinical trial, we have not observed any changes in paclitaxel pharmacokinetics, even with increasing doses of bryostatin-1. Although paclitaxel pharmacokinetics were not studied in the animals, these clinical results would suggest that a change in paclitaxel pharmacokinetics by bryostatin-1 would not account for the antagonism or enhancement observed with bryostatin-1 on the paclitaxel-treated mouse mammary tumors. Also, there are currently no available methods to measure bryostatin-1 levels in the plasma. Therefore, it is not possible to determine whether the doses of bryostatin-1 that we are using in the animals are comparable to those that are achievable in patients. To date, we have observed clinical activity with manageable toxicity in patients with recurrent esophageal and pancreatic cancer. These exciting clinical results represent a new direction in cancer therapy and illustrate the importance of the cell cycle, energy metabolism, and blood flow in the development of bryostatin-1 for clinical trials.

### REFERENCES


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**Table 3** Effect of bryostatin-1 on the rate of Gd-DTPA uptake and signal intensity

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>80 μg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>Maximum slope</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rim</td>
<td>230.8 ± 15.9</td>
<td>127.6 ± 20.1*</td>
</tr>
<tr>
<td>Center</td>
<td>166.9 ± 27.6</td>
<td>116.9 ± 37.0</td>
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<tr>
<td>Whole tumor</td>
<td>168.1 ± 23.1</td>
<td>93.8 ± 10.8b</td>
</tr>
<tr>
<td>Maximum % increase in intensity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rim</td>
<td>246.3 ± 22.8</td>
<td>187.5 ± 20.8</td>
</tr>
<tr>
<td>Center</td>
<td>218.4 ± 24.6</td>
<td>159.3 ± 19.2</td>
</tr>
<tr>
<td>Whole tumor</td>
<td>202.6 ± 21.6</td>
<td>145.2 ± 7.7</td>
</tr>
</tbody>
</table>

* P < 0.005 compared with control cohort.

b P < 0.03 compared with control.


The *in Vivo* Effect of Bryostatin-1 on Paclitaxel-induced Tumor Growth, Mitotic Entry, and Blood Flow

Jason A. Koutcher, Monica Motwani, Kristen L. Zakian, et al.


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