Preclinical Pharmacology of the Natural Product Anticancer Agent Bryostatin 1, an Activator of Protein Kinase C

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ABSTRACT

Bryostatin 1, a natural product anticancer agent isolated from a marine bryozoan, has been shown in tissue culture to activate protein kinase C. This agent has recently undergone Phase I testing in humans given either as a bolus i.v. injection or a continuous infusion. To understand how bryostatin 1 might be used best as an anticancer agent, a study of the pharmacokinetics, tissue distribution, metabolism, and elimination of bryostatin 1 in mice was undertaken, using [C26-3H]labeled bryostatin 1. Following i.v. administration, the plasma disappearance curve for bryostatin 1 could be described by a two-compartment model, with half-lives of 1.05 and 22.97 h, respectively. In contrast, the plasma disappearance curve for bryostatin 1 administered p.o. was better described by a first order absorption one-compartment model, with an absorption half-life of 0.81 h and an elimination half-life of 28.76 h, respectively. The majority of radioactivity in plasma was associated with the intact drug for up to 24 h after dosing. In the first 12 h after i.v. administration, urinary excretion represented the major pathway of elimination, with 23.0 ± 1.9% (mean ± SD) of the administered dose excreted. Within 72 h after i.v. administration, approximately equal amounts of radioactivity (40%) were excreted in feces compared to urine. Bryostatin 1 was widely distributed in many organs but concentrated in the lung, liver, gastrointestinal tract, and fatty tissue. The concentration in the gastrointestinal tract, along with the fecal excretion, suggests the possibility of enterohepatic circulation of this drug. In summary, this study demonstrates that bryostatin 1 is relatively stable in vivo, widely distributed but concentrated in some major tissues, and rapidly excreted first through urine and at later times through the feces. The data from this animal study should be useful in the design of future human trials with this anticancer drug.

INTRODUCTION

Bryostatin 1 (Fig. 1) is a natural product anticancer agent purified from the marine bryozoan, Bugula neritina (1). Although the exact mechanism of anticancer action of bryostatin 1 is not known, in tissue culture this compound activates protein kinase C, causes the translocation of this protein kinase to the cell membrane (2), and stimulates the phosphorylation of specific protein substrates. One possible mechanism underlying the antiproliferative activity of bryostatin 1 may be its ability to differentiate specific tumor types. Application of bryostatin 1 to freshly isolated cells from patients with acute and chronic leukemia (3, 4) and chronic lymphatic leukemia (5, 6), as well as leukemic cell lines HL-60 or U937 cells (6, 7), induces the differentiation of these cells and inhibits their growth. The inhibition of cell growth by bryostatin 1 is correlated with the induction of the WAF1/CIP1 protein and the dephosphorylation of cyclin-dependent kinase 2 (8). Bryostatin 1 could inhibit tumor growth by other mechanisms. For example, bryostatin 1 could stimulate the release of hormones that block tumor growth. Tumor necrosis factor-α has been shown to be released after bryostatin 1 injection (9, 10), both in humans and in mice. Another potential mechanism of anticancer activity might involve the modulation of the immune system. Bryostatin 1 stimulates interleukin 2 receptor expression on CD4+ and CD8+ lymphocytes and greatly enhances the efficiency of recombinant interleukin 2 in triggering the development of in vivo primed CTLs (11).

In mice, bryostatin 1 has anticancer activity against P388 leukemia (12), B16 melanoma (13), and M5076 ovarian sarcoma (12). The degree of inhibition of tumor growth is related to the dose, route, and schedule of bryostatin 1 administration. Mice carrying B16 melanoma and receiving multiple doses of bryostatin 1 i.p. survived longer than those which received a single dose. For those which received an equal number of doses, mice which received larger doses of bryostatin 1 (1 μg/injection) survived longer than those which received lower doses (0.5 μg/injection; Ref. 14). These data in murine tumor models demonstrate that it is likely that in humans the dosage and the schedule by which bryostatin 1 is administered will determine its anticancer activity.

Recently, bryostatin 1 has undergone Phase I trials in humans administered the anticancer agent in a number of different schedules. In one study (14), bryostatin 1 was infused over 1 h every 2 weeks. Although a dose of 50 μg/m2 was well tolerated, significant myalgia occurred at 65 μg/m2, suggesting that this latter dose was the maximally tolerated dose. This myalgia was not associated with either changes on EMG or on muscle biopsy. Other observed side effects included a transient drop in WBC and platelet counts, which returned after 4 h, fever, and lethargy. The second Phase I study of this agent infused bryostatin 1 over 1 h at doses of 25 μg/m2 every week, 35 μg/m2 every other week, or 25 μg/m2 every three weeks of every four. In this study, the dose-limiting toxicity was myalgia (9). Administering bryostatin 1 three of four weeks every month appeared to be the best tolerated schedule. Plasma levels of both interleukin 6 and tumor necrosis factor-α were elevated in patients receiving the highest dose of bryostatin 1 (9). To take advantage of the potential of bryostatin 1 to slowly induce the tumor cell differentiation and up-regulation of specific hormone receptors, additional Phase I trials of continuous infusion bryostatin 1 given are currently under way.

To better understand how bryostatin 1 can be used clinically to treat cancer patients, we investigated the preclinical pharmacology of bryostatin 1 in mice by using [C26-3H]labeled bryostatin 1 following i.v. and i.p. administration. The plasma pharmacokinetics, tissue distribution, metabolism, and elimination of bryostatin 1 were determined. The results from this study should be useful in planning future human trials.

MATERIALS AND METHODS

Preparation of [C26-3H]labeled Bryostatin 1. [C26-3H]labeled bryostatin 1 was prepared, purified, and concentrated by using the methods described previously (15).
Animals. Young adult CD1/F2 female mice (20 ± 3 g) were purchased from Charles River Laboratory. The animals were fed with commercial diet and water for 1 week prior to the study. Twenty-seven mice were assigned to 9 groups of 3 each for either i.v. or i.p. injection.

Kinetic and Tissue Distribution Study. [C26-3H]-labeled and unlabeled bryostatin 1 was dissolved in phosphate buffer containing 30% DMSO at a concentration of 40 µg/ml with ³H specific activity of 5.2 µCi/µl. Mice were administered the test compound by i.v. injection into the caudal tail vein or i.p. (0.1 ml/10 g) at the dose level of 40 µg/kg body weight. Doses were based on the pretreatment body weight and rounded to the nearest 0.01 ml. After i.v. injection, each animal was placed in a metabolism cage and fed with commercial diet and water ad libitum. Total voided urine was collected, and feces samples were homogenized in a 9-fold volume of 0.9% NaCl saline prior to quantitation of radioactivity. Blood samples were collected in heparinized tubes, and plasma was separated by centrifugation.

Animals were euthanized by exsanguination under sodium pentobarbital anesthesia, and the tissues were collected from each animal. Each tissue/organ was immediately blotted on Whatman No. 1 filter paper, trimmed of extraneous fat or connective tissue, emptied and cleaned of all contents, and weighed. Prior to homogenization in 0.9% NaCl saline (3–5 ml of wet weight), each tissue/organ was washed using 0.9% NaCl saline. The resultant homogenates were kept at −70°C until further analyses. The tissues examined were liver, kidneys, spleen, lungs, heart, thymus, brain, eyes, bone marrow, skeletal muscle, skin, lymph nodes, pancreas, stomach, small intestine, and large intestine as well as fatty tissue.

Quantitation of Total Radioactivity in Biological Samples. The total radioactivity in tissues and body fluids was determined by liquid scintillation spectrometry (LS 6000TA; Beckman, Irvine, CA), using methods described previously (15, 16). Biological fluids (plasma, 50–100 µl; urine, 50–100 µl) and cagerinse (200 µl) were mixed with 6 ml scintillation solvent (Beckman, Irvine, CA) to determine total radioactivity. An aliquot of tissue or feces homogenate (100 µl) was mixed with 0.5 ml tissue solubilizer (TS-2; Research Products International Corp., Mt. Prospect, IL); then the scintillation solvent was added (6 ml), and total radioactivity measured.

HPLC Analysis. Plasma samples (100 µl) were mixed with 2 ml of methanol to precipitate protein. After vortexing, the samples were centrifuged at 4000 rpm for 15 min. The supernatant was removed, dried under nitrogen, and then resuspended in 200 µl methanol for HPLC injection. Tissue homogenates (1 ml) were centrifuged at 14,000 rpm for 60 min at 4°C, and the supernatant was removed, mixed with 5 ml methanol, and extracted, as described above.

Reversed-phase HPLC was used to determine the metabolic profile of bryostatin 1 in plasma, liver, kidney, lung, and urine. HPLC analysis was carried out using a Beckman Gold liquid chromatography system with model 126 pumps, fraction collector or flow meter, and model 168 UV detector at wavelengths of 265 and 230 nm (Beckman, Fullerton, CA). The mobile phase included two solvents. Solvent A was doubly distilled H₂O, and solvent B was 100% acetonitrile. The column was eluted at a flow rate of 1.0 ml/min using the following gradients: (a) 0–15 min, 15%; (b) 15–19 min, 30–100%; and (c) 19–21 min, 100%. Eluent was collected every 1 min into scintillation vials and mixed with 5 ml scintillation solvent. The radioactivity was determined by liquid scintillation counting (Beckman LS 6000). Under the above conditions, the retention time of the intact drug was approximately 17.5 min with both radiochromatography and UV detection.

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**Table 1** *Plasma pharmacokinetic parameters for bryostatin 1*<sup>a</sup>

<table>
<thead>
<tr>
<th>Route</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (ng/ml)</th>
<th>T&lt;sub&gt;1/2A&lt;/sub&gt; (h)</th>
<th>T&lt;sub&gt;1/2α&lt;/sub&gt; (h)</th>
<th>T&lt;sub&gt;1/2β&lt;/sub&gt; (h)</th>
<th>AUC (ng/ml·h)</th>
<th>MRT (h)</th>
<th>VD&lt;sub&gt;ss&lt;/sub&gt; (ml/kg)</th>
<th>CL (ml/kg·h)</th>
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<tbody>
<tr>
<td>i.v.</td>
<td>92.94</td>
<td>0.81</td>
<td>28.76</td>
<td>42.19</td>
<td>2.26</td>
<td>2.37</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>i.p.</td>
<td>13.52</td>
<td></td>
<td></td>
<td></td>
<td>376.7</td>
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</table>

<sup>a</sup> Values are means based on the experimental data from 27 rats in each group following administration of [C26-³H]-labeled bryostatin 1. Concentrations in various tissues were based on the quantitation of radioactivity.

<sup>b</sup> C<sub>max</sub>, maximal concentration; T<sub>1/2A</sub>, absorption half-life; T<sub>1/2α</sub>, distribution half-life; T<sub>1/2β</sub>, elimination half-life; MRT, mean residue time; VD<sub>ss</sub>, volume of distribution; CL, clearance.
Data Analysis. The bryostatin 1 concentration was calculated based on the quantitation of radioactivity in biological fluids and tissues and was expressed as bryostatin 1 equivalents (based on the specific activity of administered [C26-3H]-bryostatin 1) in either ng/ml of biological fluids (e.g., plasma and urine) or ng/g of wet tissue/organ. Five determinations for each sample were conducted, and the concentration was then calculated. The final concentration for each time point was expressed as mean ± SD of the group of three animals.

The pharmacokinetic parameters of bryostatin 1 distribution were estimated by using the NLIN procedure of SAS programs (16–18). Functions consisting of the sum of one-, two-, or three-exponential components:

\[ C_t = \sum A_i e^{-K_i t} \]  

(\( n = 1, 2, 3 \)) were fitted to data by a least squares method, where \( C_t \) is the concentration at time point \( t \), \( A_i \) is the concentration coefficient, and \( K_i \) is the elimination or absorption rate coefficient. Selections of models were based on comparison of Akaike’s Information Criterion and SE of estimated parameters. One-, two-, and three-compartment models of i.v. bolus injection or first-order absorption administration models were tested to fit for each plasma concentration-time profile.

The AUC was calculated from:

\[ \sum A_i / K_i \]  

The elimination half-life (\( T_{1/2B} \)) values of bryostatin 1 were calculated from 0.693/\( K_i \). The clearance rate of bryostatin 1 was calculated by dividing the dose by the AUC (18).

RESULTS

Pharmacokinetics and Stability of Bryostatin 1 in Plasma. The pharmacokinetics of bryostatin 1 was evaluated following both i.v.
and i.p. administration of bryostatin 1 to mice at a dose of 40 μg/kg having a specific activity of 5.2 μCi/ml. The mean plasma concentrations over time of the bryostatin 1 following either i.v. or i.p. bolus administration of [3H]bryostatin 1 are illustrated in Fig. 2. Pharmacokinetic analysis revealed that following i.v. administration, the plasma disappearance curve for bryostatin 1 could be best described by a two-compartment model, with half-lives of 1.05 and 22.96 h (Table 1). To determine whether bryostatin 1 was degraded or modified in the plasma, the elution profile from HPLC of bryostatin 1 in the plasma was compared to that of standard bryostatin 1. These results demonstrate that 12 h after injection, bryostatin 1 in the plasma elutes at a similar location to that of control. Under reversed-phase HPLC conditions, a single major metabolite is present at 9.50 min, and other minor metabolites are present at varying times (Fig. 3).

Following i.p. administration, pharmacokinetic analysis revealed that the plasma disappearance curve for bryostatin 1 could be best described by a first-order absorption one-compartment model, with an absorption half-life of 0.81 h and an elimination half-life of 28.76 h, respectively (Table 1). Although the maximum plasma concentration of bryostatin 1 was lower after i.p. administration than that following i.v. administration, the AUC i.p. was greater than AUC i.v., indicating that bryostatin 1 was well absorbed, distributed into and retained in tissues, and relatively slowly excreted after i.p. injection compared to i.v. injection (Fig. 2; Table 1).
Elimination of Bryostatin 1 through Urine and Feces. Following i.v. administration of \[^3H\]bryostatin 1, urinary excretion represented the major pathway for elimination of bryostatin 1 in the first 12 h, with 19.28 ± 0.61% (mean ± SD) of the administered dose excreted within 6 h and 22.98 ± 1.85% within 12 h (Fig. 4). In the first 12 h after dosing, fecal excretion was the minor pathway for eliminating bryostatin 1. However, within 72 h after dosing, fecal excretion became the major pathway, with an approximately equal amount of radioactivity excreted in feces as in urinary excretion (43.16 ± 2.74% versus 36.48 ± 5.0%). This excretion profile suggests the possibility that bryostatin-1 may undergo enterohepatic circulation.

Following i.p. administration, 13.4 ± 4.7% of the administered dose was excreted through urine within 12 h with 35 ± 5.1% excreted within 72 h. Significant fecal excretion was also observed, with 17.7 ± 1.32% excreted within 24 h and 28.4 ± 7.6% within 72 h (Fig. 4). The total excretion of bryostatin 1 following i.p. administration was slightly lower than that observed with i.v. injection (63% versus 80% within 72 h after administration). The excretion kinetics correlated with plasma concentration changes detailed above (Table 1; Fig. 2).

Distribution and Stability of Bryostatin 1 in Various Tissues. In this study, stability and distribution of bryostatin 1 were determined in various tissues between 5 min and 72 h following i.v. administration and between 1 and 72 h after i.p. administration.

As illustrated in Fig. 5, bryostatin 1 had a wide tissue distribution, with detectable radioactivity in all the tissues examined after i.v. bolus administration. To clearly describe the tissue distribution pattern of bryostatin 1, the mean bryostatin 1-equivalent concentrations of radioactivity in various tissues were plotted along with corresponding plasma concentrations (Fig. 5). In the initial 30 min after dosing, bryostatin 1 was distributed in kidneys, liver, spleen, bone marrow, and the gastrointestinal tract, with the highest concentration of bryostatin 1 being found in the liver, lung, and bone marrow. Most tissues had significantly higher concentrations than did plasma 4 h and longer after dosing. In vivo stability of bryostatin 1 in various tissues was evaluated by HPLC, demonstrating that bryostatin 1 was largely intact in most tissues, e.g., the liver, kidneys, and lung (Fig. 6). However, in both the kidney and the liver, a single major metabolite similar to that seen in the blood was apparent.

A similar pattern of tissue distribution of bryostatin 1 was observed following i.p. administration (Fig. 7), suggesting that bryostatin 1 was taken up by all tissues. At 6 h and longer after i.p. administration, the concentrations of bryostatin 1 in most tissues were similar to or higher than those observed with i.v. injection at similar times. A significant accumulation of radioactivity in fatty tissue was also observed (Fig. 7C). The observed greater concentrations in tissues after i.p. injection reflect the differences in elimination between i.p. and i.v. administration, i.e., slower excretion of bryostatin 1 following i.p. administration than after i.v. administration. The stability of bryostatin 1 in various tissues following i.p. administration was further evaluated by HPLC, demonstrating that, like i.v. administration, largely intact bryostatin 1 with a single major metabolite was present in most tissues, e.g., the kidneys, liver, and lung (data not shown).

**DISCUSSION**

In human clinical trials, bryostatin 1 has been administered either as a repeated 1 h bolus or as a continuous infusion over 72 h. To predict the best dose and schedule for future clinical evaluation of this drug, the fundamental pharmacokinetic parameters and the possible differences between i.p. and i.v. administration have been evaluated in a murine model system.

Pharmacokinetic analysis reveals that following i.v. administration, the plasma disappearance curve for bryostatin 1 could be best described by a two-compartment model, with half-lives of 1.05 and 22.96 (Table 1). The shorter half-life illustrates that bryostatin 1 was rapidly distributed to other compartments (tissues) outside the plasma, and the relatively longer elimination half-life reflects the retention of bryostatin 1 in the body. Urinary excretion represented the major pathway of elimination of bryostatin 1 for the first 12 h after i.v. administration, with fecal excretion, a minor pathway during this initial period, becoming a major elimination pathway 12 h after administration. Following i.p. administration, pharmacokinetic analysis revealed that plasma disappearance curves for bryostatin 1 could be best described by a first-order absorption one-compartment model, with an absorption half-life of 0.81 h and an elimination half-life of 28.76 h, respectively (Table 1). The elimination half-life following i.p. administration was longer than that of i.v. administration, indicating that bryostatin 1 was retained in the body for a longer period following i.p. administration than following i.v. injection. Other pharmacokinetic parameters, as illustrated in Table 1, support this conclusion, i.e., greater AUC, longer mean residue time, and lower clearance with i.p. administration than with i.v. administration. Closer examination of urinary and fecal excretion of bryostatin 1, following both i.v. and i.p. administrations, indicate that the longer retention of bryostatin 1 in the body after i.p. administration is caused by a slower excretion, especially within the first 12 h. Thus, because i.p. injection avoids rapid renal excretion, it is an alternative route of administration.

Following both i.v. and i.p. administrations of bryostatin 1, there was a wide distribution of radioactivity in various tissues, including the liver, bone marrow, lung, spleen, kidney, and gastrointestinal tract. A significant accumulation of bryostatin 1 in fatty tissue was observed with i.p. administration, which may be related to the delayed elimination of bryostatin 1 and, therefore, may be important to the biological effects of this drug. HPLC analysis of tissue extracts demonstrates that the compound is largely intact. However, metabolites were identified by reverse-phase HPLC analysis, which have shorter retention times than intact bryostatin 1. Further studies to identify these metabolites are in progress. Bryostatin 1 appears to be concentrated in the...
liver and gastrointestinal tract, suggesting the possibility of enterohepatic circulation. Also, high concentrations of this drug were found in the lung, suggesting the possibility that bryostatin 1 may be active at this location as well.

Although it is not always possible to extrapolate data from animals to humans because of potential species differences, this study suggests that because bryostatin 1 had a short distribution half-life and a relatively prolonged elimination half-life (about 24 h), it can be best administered as an i.v. loading dose, followed by a maintenance dose every other day. Significant accumulation in the liver and the gastrointestinal tract, as well as fecal excretion of bryostatin 1, indicate that an enterohepatic circulation of bryostatin 1 (and possibly its metabolites) may occur. Therefore, careful monitoring of liver functions and possible gastrointestinal side effects will be necessary. The higher tissue levels of bryostatin 1 after i.p. administration suggest that in cases where this route is practical, the distribution in the tissues is better from i.p. administration than from i.v. administration. Additional pharmacological analysis of human samples obtained after bryostatin 1 chemotherapy should shed further light on how bryostatin 1 can be best administered to achieve maximal biological effect.

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