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A sensitive platelet activation-based functional assay for the antileukemic agent bryostatin 1

Marcus E Carr, Jr,[1–3,5,6] Sheryl L Carr[4,6] and Steven Grant[1–5]

[1]Division of Hematology/Oncology, Departments of [2]Internal Medicine, [3]Pharmacology and Toxicology, and [4]Pathology, [5]Massey Cancer Center and [6]Coagulation Special Studies Laboratory, Medical College of Virginia, Richmond, VA 23298, USA. [7]McGuire VA Medical Center, Richmond, VA 23249, USA.

Bryostatin 1, a macrocyclic lactone activator of protein kinase C (PKC) currently in phase I evaluation, is a biologic response modifier which exhibits significant antitumor activity in several experimental systems. Clinical trials have been hampered by the absence of a sensitive assay for bryostatin 1 blood levels. The purpose of these studies was to exploit the exquisite sensitity of human platelets to bryostatin 1-induced aggregation in order to develop an assay capable of detecting plasma bryostatin 1 levels in the nanomolar range. Addition of bryostatin 1 (5–100 nM) to platelet-rich plasma resulted in complete platelet aggregation. A highly linear relationship was observed between low bryostatin 1 concentrations (i.e. 2–25 nM) and (i) reduction in the lag phase prior to aggregation and (ii) maximal rate of aggregation (R = 0.976). At higher bryostatin 1 concentrations (i.e. 10–100 nM), platelet aggregation was accompanied by detectable ATP release; both the extent and maximal rate of ATP secretion were highly linear functions of bryostatin 1 levels (R = 0.992). Bryostatin 1 concentrations in anticoagulated human blood samples could also be determined by mixing platelet poor plasma obtained from such samples with normal platelet-rich plasma. Notably, measurement of the delay in the aggregation lag phase permitted quantitation of bryostatin 1 concentrations of 5 nM or below. The capacity to detect bryostatin 1 plasma levels of 10 nM or lower should facilitate the conduct of pharmacokinetic and pharmacodynamic studies in conjunction with ongoing phase 1 trials.

Key words: Bryostatin 1, platelet activation, platelet aggregation, platelet secretion, protein kinase C.

Introduction

Bryostatin 1, a macrocyclic lactone activator of protein kinase C (PKC), has multiple interesting biological, antitumor and immunomodulatory activities.[1] Unlike other PKC activators such as phorbol myristate acetate (PMA), bryostatin 1 lacks tumor-promoting capabilities; instead, it blocks certain phorbol-mediated actions that it does not itself possess.[2,3] In *in vitro* studies, bryostatin 1 has been shown to activate neutrophils[4] and to induce differentiation in peripheral blood chronic myelogenous leukemia cells,[5] as well as in several malignant myeloid[6] and lymphoid[7] cell lines. Evidence of antitumor selectivity has emerged from studies demonstrating that bryostatin 1 stimulates the growth of normal hematopoietic progenitors,[8] possibly by an indirect mechanism,[9] while inhibiting the clonogenic capacity of their leukemic counterparts.[10] Bryostatin 1 may also function as a biological response modifier, potentiating the activity of hematopoietic growth factors such as recombined granulocyte macrophage colony stimulating factor both *in vitro*[11] and *in vivo*,[12] and enhancing ara-C-induced apoptosis in the human promyelocytic leukemia cell line HL-60.[13] Finally, bryostatin 1 has demonstrated promising preclinical activity against a variety of tumor types, including breast carcinoma,[14] melanoma,[15] lymphoma[16,17] and leukemia.[18] Together, these properties have prompted the initiation of phase I studies of the compound in human malignancy.[19,20]

A major impediment to the conduct of pharmacokinetic studies in conjunction with such trials is the absence of a rapid and sensitive assay for detection of bryostatin 1 blood levels. This is particularly important in the case of an agent such as bryostatin 1, whose actions are highly dose-dependent and potentially manifested at extremely low concentrations (i.e. 1–10 nM).[11,13] Unfortunately, attempts to develop a highly sensitive HPLC assay for this compound have not as yet been successful. Using a neutrophil activation assay, Berkow and coworkers attempted to measure bryostatin 1 levels in the plasma of C57BL/6 mice injected with 0.01–1.0 μg bryostatin 1.[21] They found that plasma bryostatin 1 levels declined to a value below the level of

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Correspondence to ME Carr, Box 980230 MCV Station, Department of Medicine, Medical College of Virginia, Richmond, VA 23298, USA. Tel: (+1) (804) 828-7557; Fax: (+1) (804) 828-8079

detection of their assay (i.e. 60 nM) within minutes after injection. Two phase I trials have recently been completed in which bryostatin 1 was administered as a weekly 1 h infusion.[19,20] Although toxicity data were obtained and a maximum tolerated dose (MTD) for bryostatin 1 identified, the lack of a suitable assay method made pharmacokinetic analysis impossible. Consequently, there is currently a need for a sensitive and reliable method for estimating plasma bryostatin 1 levels in patients enrolled in ongoing trials of this agent.

A possible solution to this problem lies in the observation that human platelets contain significant amounts of PKC.[22] Platelet PKC can be activated by diacylglycerol (DAG) and by DAG analogs such as phorbol esters.[23,24] Such activation leads to translocation of PKC from the cytosol to the membrane component.[25,26] Platelet stimulation by agonists such as collagen, thrombin and serotonin are known to involve PKC activation.[27,28] Moreover, the latter two agonists have been shown to produce translocation of PKC from cytosol to membrane.[28] Initial reports of bryostatin 7 induced aggregation of human platelets[29] were followed by studies indicating that bryostatin 1 potently induced platelet aggregation,[21,30,31] platelet dense granule secretion[21,30] and alterations in platelet morphology.[31] The present study was prompted by the hypothesis that the exquisite sensitivity of platelets to bryostatin 1 could be exploited to develop a simple yet sensitive bioassay capable of detecting the very low levels of this compound that might be achievable *in vivo*. Our findings confirm the pronounced platelet aggregating properties of bryostatin 1, and demonstrate the feasibility of exploiting this phenomenon in order to measure plasma bryostatin 1 concentrations in the nanomolar range.

Materials and methods

Materials

Bryostatin 1 (lot no. 91-208; Ben Venue, Bedford, OH) was supplied by the Division of Cancer Treatment, National Cancer Institute (NIH, Bethesda, MD) and was reconstituted with the accompanying PET diluent (60% polyethylene glycol, 30% dehydrated ethyl alcohol, 10% polysorbate 80 v v) to yield a 10^{-4} stock solution. Further dilution of bryostatin 1 was accomplished by the addition of 0.15 M NaCl.

Sample collection

Whole blood obtained with informed consent from normal volunteers was collected in 3.8% sodium citrate. These studies have been approved by the Human Investigations Committee of the Medical College of Virginia. Platelet-rich plasma was prepared by centrifugation for 5 min at 500 g. Platelet-poor plasma was prepared by additional centrifugation at high speed (e.g. 20 000 *g*) for 20 min.

Measurement of platelet ATP secretion in platelet-rich plasma

ATP release was measured utilizing Chrono-Lume® reagent (Chronolog, Havertown, PA) and a Chrono-Log® (Chronolog) whole blood lumi-aggregometer using a previously described method.[32] For each assay, 900 µl of platelet-rich plasma (platelet count = 200 000/µl) and 100 µl of Chrono-Lume reagent were placed in an aggregometer cuvette equipped with a stirring bar. The amount of ATP secreted was determined by comparing the peak luminescence signal for a test sample to luminescence signals generated by known amounts of a Chrono-Lume ATP standard.

Measurement of bryostatin 1 mediated release of platelet ATP

The effects of bryostatin 1 were measured by addition of increasing amounts of bryostatin 1 to platelet-rich plasma and subsequent monitoring of ATP release. Since the bryostatin 1 utilized in this study was dissolved in PET, possible effects of PET were excluded by appropriate control measurements.

Measurement of platelet aggregation in platelet-rich plasma

Platelet aggregation was measured utilizing a Chrono-Log® whole blood lumi-aggregometer as previously described.[32,33] Platelet-rich plasma (900 µl) was placed in an aggregometer cuvette equipped with a stirring bar. Platelet concentration was set to 200 000 µl by diluting platelet-rich plasma with platelet-poor plasma. Once a stable baseline was obtained, 100 µl of bryostatin 1 test solution was added. Platelet aggregration was monitored electrically as an increase in impedance

(ohms). The effects of bryostatin 1 were measured by addition of increasing amounts of bryostatin 1 to platelet-rich plasma at time zero and subsequently monitoring platelet aggregation. As described above, possible effects of the PET diluent were excluded by appropriate control measurements. In several experiments, platelet aggregation was monitored optically as an increase in light transmission (%). In these measurements, 50 µl of bryostatin 1 in solution (or plasma) were added to 450 µl of platelet-rich plasma.

Measurement of bryostatin 1 in human blood samples

Bryostatin 1 was added to samples of normal human blood and the mixture was allowed to incubate at room temperature for 5 min. Platelet-poor plasma was then prepared as described above. Bryostatin containing plasma (600 µl) was subsequently utilized to induce platelet aggregation in 400 µl of normal platelet-rich plasma. Platelet-poor plasma lacking bryostatin 1 served as the baseline control. Platelet aggregation induced by test samples demonstrated concentration-dependent lag phases prior to the onset of aggregation. Lag phase times were defined as the intercept of tangents to the baseline and maximum rate of rise portions of the aggregation curves. Lower bryostatin 1 concentrations were associated with longer lag phases. The bryostatin concentration for each sample was determined based on the demonstrated relationship between bryostatin 1 concentration and the duration of the lag phase.

Statistical analysis

The Student's *t*-test for unpaired observations was employed to determine the significance of differences between experimental values.

Results

The ability of bryostatin 1 to induce platelet aggregation is demonstrated in Figures 1–4. Figure 1 depicts the kinetics of bryostatin 1 induced platelet aggregation in normal human plasma as measured optically. After a 60 s lag phase, 100 nM bryostatin 1 induced rapid, complete aggregation. At a 50 nM bryostatin 1 concentation, the lag phase increased to 84 s and the maximal rate of aggregation declined

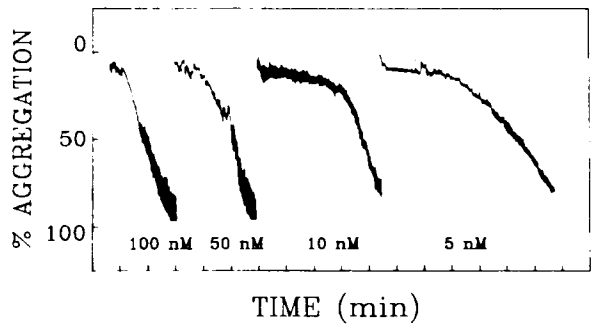

Figure 1. Optical measurement of bryostatin 1 induced platelet aggregation. Bryostatin 1 was added to anticoagulated platelet-rich plasma at time zero. Platelet concentration was 200 000/µl and final bryostatin 1 concentrations were as indicated. A representative experiment is shown; two additional studies yielded equivalent results.

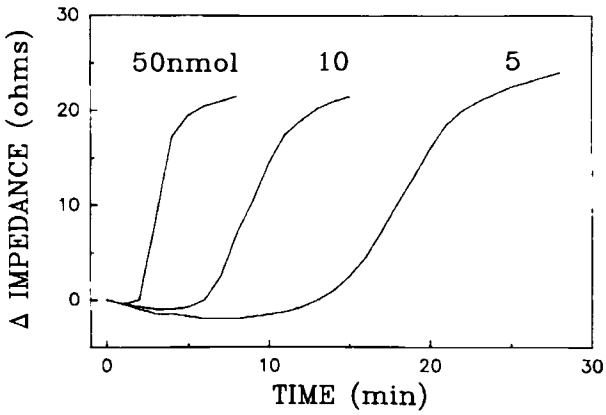

Figure 2. Kinetics of impedance changes during bryostatin 1 induced aggregation. Bryostatin 1 at the indicated concentrations was added at time zero. Platelet concentration was 200 000/µl. A representative study is shown; two additional experiments yielded equivalent results.

by 40%, but the extent of aggregation was unaltered. At 10 and 5 nM bryostatin 1 levels, the lag phase was extended to above 500 s and the maximum rate of aggregation displayed a further decline. The effects of bryostatin 1 concentration on platelet aggregation could also be monitored by changes in electrical impedance (Figure 2). The lag phase prior to aggregation became progressively prolonged as the bryostatin 1 concentration declined. While the extent of aggregation was equivalent in all cases, the maximal rate of aggregation declined significantly (i.e. from 10.9 to 4.6 to 2.3 ohms/min) as the bryostatin 1 concentration was reduced from 50 to 10 to 5 nM, respectively ($p \leq 0.05$ in each case; Figure 3). A highly linear inverse relationship was noted between the maximal platelet aggregation rate and bryostatin 1 concentrations as low as

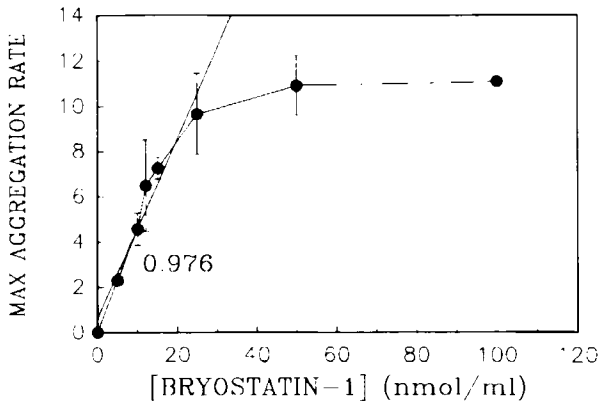

Figure 3. Effect of bryostatin 1 concetration on the maximal aggregation rate (ohm/min) as determined by impedance measurement. Maximal aggregation rates were defined as the slope of a tangent drawn to the steepest portion of the impedance curve. Each data point represents the mean ± SEM of three measurements. Two additional studies yielded similar results.

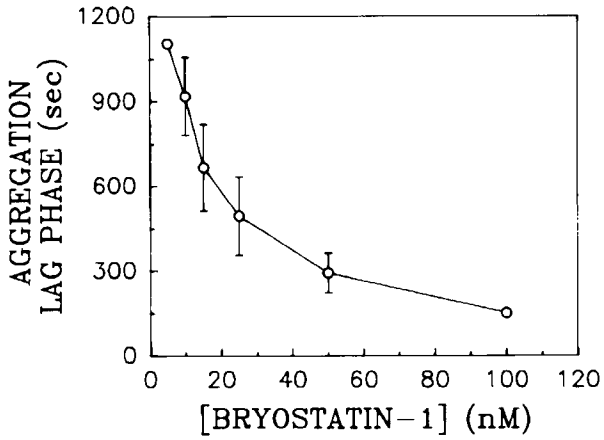

Figure 4. Effect of bryostatin 1 concentration on aggregation lag phase in seconds. Time zero was defined as the moment of bryostatin addition. Each data point represents the mean ± SEM of three measurements. A representative study is shown; three additional experiments yielded similar results.

5 nM ($R = 0.976$). The relationship between aggregation lag phase and bryostatin 1 concentration was defined further by examining the effects of a broad range of bryostatin 1 concentrations (e.g. 5–100 nM) on the platelet aggregation lag phase (Figure 4). Below 20 nM bryostatin 1, lag phases became significantly prolonged ($p \leq 0.05$) with declining bryostatin 1 concentration. At bryostatin 1 concentations of 50 nM or above, the rate of change in lag phase with increasing bryostatin concentration was considerably less pronounced. Nevertheless, at bryostatin 1 levels of 25 nM or lower, there was a highly linear relationship between concentration and aggregation lag phase ($R = 0.996$). In fact, linearity was preserved at bryostatin 1 levels as low as 2 nM (data not shown). Thus, these findings suggest that assessment of maximal platelet aggregation rate and aggregation lag phase can be used to quantify bryostatin 1 levels as low as 2–5 nM.

The effects of bryostatin 1 on ATP secretion by normal human platelets are demonstrated in Figures 5–7. Figure 5 depicts the kinetics of ATP release from platelets following addition of the indicated concentrations of bryostatin 1 at time zero. Measurable increases in ATP release were observed for

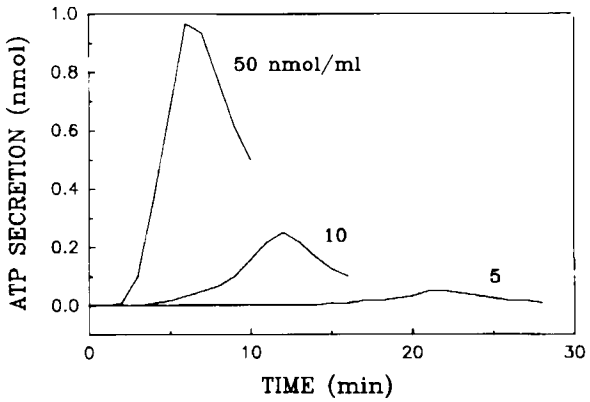

Figure 5. Bryostatin 1 induced ATP secretion from normal human platelets during bryostatin induced platelet aggregation in platelet-rich plasma. Bryostatin at the indicated concentration was added at time equal zero. Platelet concentration was 200 000/μl. Single, representative curves are shown. Three additional studies yielded comparable results.

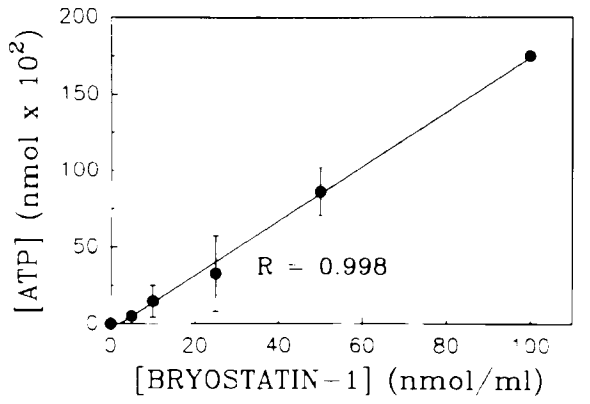

Figure 6. Peak amount of ATP secreted from human platelets as a function of bryostatin 1 concentration. ATP concentration was calculated from data obtained as demonstrated in Figure 5. Each data point represents the mean ± SEM of three measurements. Two additional studies yielded equivalent results.

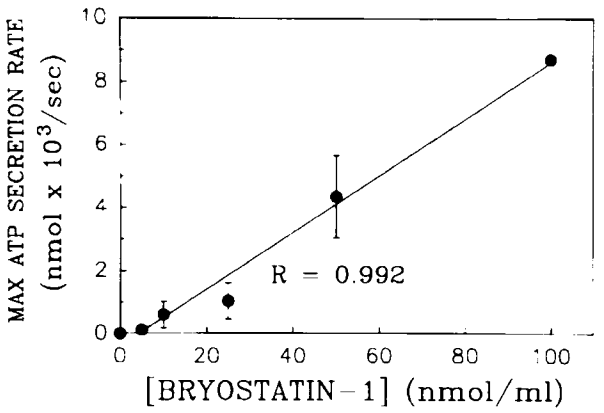

Figure 7. Effect of bryostatin 1 concentration on the maximum rate of ATP secretion from platelets. Maximum rate was calculated as the slope of a tangent to the steepest portion of ATP secretion curves such as depicted in Figure 5. Each data point represents the mean ± SEM of three measurements. The study shown is representative of three similar experiments.

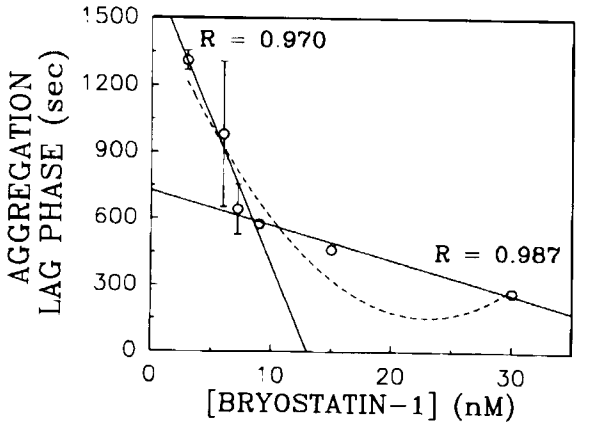

Figure 8. Induction of platelet aggregation by mixing normal platelet-rich plasma with platelet-poor plasma containing bryostatin 1. Time zero was defined as the moment of mixing. Each data point represents the mean ± SEM of three measurements. Two linear regressions are drawn for plots through data above and below a bryostatin 1 concentration of 7.5 nM. Regression coefficients for these plots are as indicated. The dotted line is a second-order regression to the entire data set ($R = 0.948$). An additional study yielded equivalent results.

bryostatin 1 concentrations above 5 nM. Dose–response analysis of these curves revealed a strikingly linear relationship between both the extent (Figure 6) and the rate (Figure 7) of ATP secretion and the bryostatin 1 concentration ($R = 0.998$ and 0.992, respectively). In contrast to the platelet aggregation assays described above, linearity was maintained throughout the entire 10–100 nM bryostatin 1 test concentration range. A strong, albeit non-linear relationship also existed between the lag phase prior to secretion and the bryostatin concentration (data not shown). Thus, measurement of platelet ATP secretion provides an alternative method for quantifying bryostatin 1 concentrations in the 10–100 nM range.

An attempt was then made to determine if these assays could be used to detect bryostatin 1 in whole blood samples and the results are shown in Figure 8. In these experiments, varying concentrations of bryostatin 1 were added to anticoagulated whole blood obtained from normal donors. Platelet-poor plasma was prepared from these samples and subsequently mixed with normal platelet-rich plasma. Addition of bryostatin 1 to the test samples induced concentration-dependent platelet aggregation and ATP secretion. As noted in the case of direct addition of bryostatin 1 to plasma (Figure 4), the lag phase prior to initiation of aggregation continued to be directly related to the amount of bryostatin 1 present in the test sample (Figure 8). The indicated bryostatin 1 concentrations were calculated from the amount of bryostatin 1 added to the original whole blood sample and corrected for dilution due to combination of the test sample with normal pooled plasma. At bryostatin 1 concentrations ranging from 7.5 to 30 nM, the lag phase declined at a rate of 15.4 s/nM bryostatin 1, and a high degree of linearity was noted ($R = 0.987$). At lower bryostatin 1 concentration (i.e. below 10 nM), the lag phase was prolonged by 132 s/nM reduction in bryostatin 1 concentration, and a similar degree of linearity was observed ($R = 0.970$), even at concentrations as low as 3 nM. Studies performed with frozen plasma samples also revealed concentration-dependent bryostatin 1 induced platelet aggregation at these levels (data not shown). At room temperature, bryostatin 1 appeared to be stable in platelt-poor plasma for at least 30 min. Luminescence measurements were routinely performed in conjunction with all aggregation assays. While ATP secretion was detected during mixing experiments, the amount of ATP secreted was too low to permit determination of bryostatin 1 concentrations below 10 nM, although at higher concentrations (i.e. 20 nM or above), linearity was preserved for bryostatin 1 concentrations as high as 100 nM ($R =$ patient sample 0.992; not shown).

Discussion

The unique activities of bryostatin 1, either as an antineoplastic agent in its own right or as a biological response modifier capable of enhancing the

efficacy of other agents, have generated considerable interest in its application to the treatment of human malignancy. Bryostatin 1 exerts *in vitro* and *in vivo* cytotoxic effects towards several malignant cell lines,[14–18] and potentiates the antileukemic activity of ara-C against human myeloid leukemia cells.[13] The latter effect appears to result from facilitation of ara-C-related apoptosis as a consequence of down-regulation of cellular PKC activity.[34] In addition, bryostatin 1 shows direct evidence of antileukemic selectivity, in that it promotes the growth of normal hematopoietic progenitors,[8,9,11] while inhibiting the growth and self-renewal capacity of their leukemic counterparts.[10,18,35] Whether these promising findings can be extended to *in vivo* studies in humans will in all likelihod depend upon the achievement of plasma bryostatin 1 levels approximating those shown to be effective in the preclinical setting. To date, clinical trials of bryostatin 1 have been limited by the absence of pharmacokinetic information. Such data are unavailable primarily due to the lack of a reproducible and sensitive assay of bryostatin 1 plasma concentrations. Previously proposed assays have monitored the reduction of cytochrome *c* by neutrophils as a marker of bryostatin 1 concentration and reportedly can detect bryostatin 1 concentrations as low as 60 nM.[21] However, in mice receiving a single intravenous injection of bryostatin 1 (1 μg), plasma concentrations quickly declined to undetectable levels,[21] possibly reflecting rapid clearance or, alternatively, extensive binding of bryostatin 1 to intracellular targets (i.e. PKC). If a similar phenomenon occurs in humans, more sensitive assays will be required if pharmacokinetic studies of this compound are to be underaken. The current findings demonstrate that human platelets are sensitive to bryostatin 1 levels as low as 5 nM (and below), and that several platelet responses, including aggregation lag phase and degree of ATP secretion, are linearly related to bryostatin 1 concentration. These relationships may permit determination of potentially very low bryostatin 1 plasma levels through measurement of the effect of test samples on platelet-rich plasma.

The ability to utilize platelet function as a marker of bryostatin 1 concentration presumably represents a consequence of inherent platelet PKC activity.[22] Platelets are rich in PKC and several platelet functions appear to depend on PKC mediated intracellular signalling. For example, collagen-induced platelet aggregation is known to depend on PKC activation.[27] In addition, platelet activation by serotonin or thrombin results in PKC activation and translocation of PKC from the cytosol to the membrane fraction.[28] Bryostatin 1 binds to platelet PKC near the PMA binding site, resulting in increased intracellular Ca^{2+} concentration, P47 phosphorylation, dense granual release[30,33] and platelet aggregation.[21,30,31,36] While bryostatin 1 induced platelet morphologic changes are not dramatic when monitored optically, electron micrographic studies have demonstrated pseudopodia formation consistent with platelet activation.[31] The present findings provide additional evidence that several platelet responses are exquisitely sensitive to bryostatin 1 action, and indicate that the linear relationship between these responses and bryostatin 1 concentrations may be exploited to estimate plasma bryostatin 1 levels. In this regard, reduction in the platelet aggregation lag phase was found to be particularly sensitive to bryostatin 1 concentration and this determinant may prove valuable in assaying the very low plasma levels (i.e. 1–10 nM) that might be achievable in the *in vivo* setting. Assays related to platelet ATP were somewhat less sensitive than those involving platelet aggregation, but offer the advantage of providing linear correlations over higher plasma bryostatin 1 concentrations (i.e. 10–100 nM). It should be mentioned that in previous studies employing a murine model, bryostatin 1 was found to inhibit thrombin induced platelet ATP secretion.[21] It is possible that this discrepancy represents a species-related difference. In any event, since bryostatin 1 is a highly potent activator of PKC, and exerts several of its effects at concentrations in the low nanomolar range,[13,18] the availability of the present assays should help to establish the *in vivo* relevance of earlier *in vitro* observations.

The effects of bryostatin 1 on platelets appear to be unique among chemotherapeutic agents. While most myelosuppressive agents cause significant thrombocytopenia, there are few reports of direct effects of chemotherapeutic agents on platelet function. BCNU has been reported to inhibit platelet aggregation by a poorly described mechanism.[37] Metabolites of cyclophosphamide, including acrolein and 4-HC, inhibit platelet aggregation by inhibiting PKC.[38] Such antiplatelet effects are thought to play a role in the increased hemorrhagic risk associated with high doses of these agents.[39] Thus, bryostatin 1 appears to be the first antineoplastic agent with platelet activating activity and this property may explain acute transient thrombocytopenia associated with bryostatin 1 administration.[19,20]

In summary, the results of the present study demonstrate that human platelet aggregation and ATP release are exquisitely sensitive to the actions of

very low concentrations of bryostatin 1, and that the linear relationship between bryostatin 1 concentrations and platelet activation-related events allows detection of bryostatin 1 levels in the low nanomolar range. In addition, platelet activation-based assays can be adapted to estimate bryostatin 1 concentrations in whole blood samples. Such assays should facilitate pharmacokinetic studies of bryostatin 1 in conjunction with ongoing phase I trials.

Conclusions

The purpose of this study was to determine whether the potent effects of the PKC activator bryostatin 1 on human platelet responses could be employed to develop a sensitive bioassay suitable for pharmacokinetic monitoring of plasma concentrations below 100 nM in conjunction with ongoing phase I trials. Our results indicate that a highly linear relationship exists between bryostatin 1 concentration and (i) the maximal rate and lag phase of platelet aggregation and (ii) the peak amount and maximal rate of platelet ATP secretion ($R \geq 0.970$ in each case). Moreover, in the case of platelet aggregation-based assays, linearity is observed at bryostatin 1 levels as low as 2–10 nM, concentrations previously shown to exhibit significant biological effects in preclinical studies. Finally, these assays can be adapted to permit estimation of bryostatin 1 concentrations in human whole blood samples. In this regard, measurement of bryostatin 1-mediated reduction in platelet aggregation lag phase may be ideally suited for determining plasma bryostatin 1 concentrations of 10 nM or lower. The availability of a sensitive platelet aggregation-based assay should permit correlative pharmacokinetic and pharmacodynamic studies to be undertaken in conjunction with ongoing phase I trials of this promising biologic response modifier.

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