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Comparison of transcriptional response to phorbol ester, bryostatin 1, and bryostatin analogues in LNCaP and U937 cancer cell lines provides insight into their differential mechanism of action

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Abstract

Bryostatin 1, like the phorbol esters, binds to and activates protein kinase C (PKC) but paradoxically antagonizes many but not all phorbol ester responses. Previously, we have compared patterns of biological response to bryostatin 1, phorbol ester, and the bryostatin 1 derivative Merle 23 in two human cancer cell lines, LNCaP and U937. Bryostatin 1 fails to induce a typical phorbol ester biological response in either cell line, whereas Merle 23 resembles phorbol ester in the U937 cells and bryostatin 1 in the LNCaP cells. Here, we have compared the pattern of their transcriptional response in both cell lines. We examined by qPCR the transcriptional response as a function of dose and time for a series of genes regulated by PKCs. In both cell lines bryostatin 1 differed primarily from phorbol ester in having a shorter duration of transcriptional modulation. This was not due to bryostatin 1 instability, since bryostatin 1 suppressed the phorbol ester response. In both cell lines Merle 23 induced a pattern of transcription largely like that of phorbol ester although with a modest reduction at later times in the LNCaP cells, suggesting that the difference in biological response of the two cell lines to Merle 23 lies downstream of this transcriptional regulation. For a series of bryostatins and analogues which ranged from bryostatin 1-like to phorbol ester-like in activity on the U937 cells, the duration of transcriptional response correlated with the pattern of biological activity, suggesting that this may provide a robust platform for structure activity analysis.

Keywords

PMA; human prostate cancer; therapeutic; human leukemia; Merle 23

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1. Introduction

The protein kinase C (PKC) isozymes represent a critical node in cellular signaling, responding to the lipophilic second messenger *sn*-1,2-diacylglycerol generated upon receptor mediated phosphoinositide breakdown [1]. Consistent with this functional role, PKC has emerged as an attractive therapeutic target both for cancer as well as for a wide range of other conditions such as diabetic retinopathy or dementia [2–4]. Therapeutic strategies directed at PKC include inhibitors of its enzymatic activity, e.g. enzastaurin [5], and agents targeted to its regulatory domain, e.g. prostratin, PEP005, and bryostatin 1 [6]. These latter agents all bind to the C1 domain of PKC, which functions as the regulatory diacylglycerol recognition motif on PKC.

Bryostatin 1 is a macrocyclic lactone initially isolated from the marine organism *Bugula neritina* on the basis of growth inhibitory activity in the P388 cell assay [7–8]. It has been extensively studied in clinical trials both as a single agent as well as in combination [9] (<http://www.clinicaltrials.gov>). Among PKC ligands, the bryostatins are unique in that, while they function *in vitro* as PKC activators, in many cellular systems they behave as PKC antagonists, blocking response to typical PKC activators such as the phorbol esters when co-administered. Of particular importance, whereas the phorbol esters provide the paradigm for tumor promoters, bryostatin 1 itself shows little promoting activity and inhibits the promoting activity of the phorbol esters [10]. Understanding the mechanism(s) underlying its functional PKC antagonism promises great impact – for developing simplified bryostatin analogs retaining its unique behavior, for designing novel compounds exploiting this/these mechanisms, and for rationally targeting bryostatin and its analogs to those cancers susceptible to this/these mechanisms.

Mechanistically, bryostatin 1 shows multiple differences compared to typical phorbol esters in how it affects PKC isoforms. The tumor promoting phorbol 12-myristate 13-acetate (PMA) causes initial localization of PKC δ to the plasma membrane followed by partial redistribution to internal membranes; bryostatin 1 causes localization of PKC δ directly to the internal membranes [11]. In many systems, PMA down regulates PKC δ ; bryostatin 1 displays a biphasic dose response curve for down regulation of PKC δ with protection at higher bryostatin 1 concentrations [12]. On the other hand, in a number of systems bryostatin 1 causes faster down regulation of PKC α and PKC β than does PMA [12–15]. In addition, PMA induces phosphorylation of PKC δ at Y311; bryostatin 1 induces much less [15].

At the level of downstream response, bryostatin 1 has often been found to induce a more transient response than does phorbol ester. In mouse epidermal cells, phorbol ester caused long lasting inhibition of cell-cell communication; inhibition by bryostatin 1 was largely lost by 3 hrs [16]. Similarly, in these cells the inhibition of epidermal growth factor binding by bryostatin 1 was lost by 4 hrs whereas it was of long duration with phorbol ester [17]. Consistently, in these and other cases the transient responses to bryostatin 1 cannot be explained by instability of the bryostatin 1, since cotreatment with bryostatin 1 and phorbol ester gave a response similar to that by bryostatin 1 alone. It should be emphasized, however, that the pattern of behavior greatly depends on the specific cell type. Thus, in C3H10T1/2 mouse fibroblasts bryostatin 1 caused persistent inhibition of epidermal growth factor binding, contrasting with its transient behavior in the mouse epidermal cells [18]. Likewise, in Swiss 3T3 cells bryostatin 1, like phorbol ester, induced proliferation, a long-term response [19].

A major obstacle to understanding the linkage between the mechanistic differences between the phorbol esters and bryostatin 1 and their different biology has been the limited diversity

of natural bryostatin analogs. The impressive strides in bryostatin chemistry over the last few years now promise to revolutionize this situation [20–23]. As an initial approach, we have examined a series of bryostatin derivatives in two systems in which PMA and bryostatin 1 have different effects. In the U937 human myeloid leukemia cell line, PMA inhibits proliferation and induces cellular attachment; bryostatin 1 fails to do so and blocks the PMA response [24–26]. In the LNCaP human prostate cancer cell line, PMA inhibits cell growth [27–28], in part as a consequence of its induction of tumor necrosis factor α [29–30]; bryostatin 1 again fails to do so and blocks the PMA response [30–32]. Strikingly, the bryostatin analog Merle 23 induced a PMA-like response in the U937 cells [33], whereas it was bryostatin-like in its effect on cell growth of the LNCaP cells [15]. More detailed analysis suggested that the behavior of Merle 23 in the LNCaP cells was complicated, however, if evaluated for a broader range of responses, with differences from bryostatin 1 in PKC isoform down regulation, localization, pattern of phosphorylation, and duration of response [15].

Building on our previous studies with Merle 23 in the U937 [33] and LNCaP cells [15], which examined a limited series of biological and biochemical endpoints, here we have sought to obtain a more comprehensive view of the comparative effects of phorbol ester, bryostatin 1, and the bryostatin 1 analog Merle 23 on the LNCaP and U937 cells. Using qPCR, we examined the effects of the compounds as a function of dose and time on the levels of mRNA expression for 18 genes. These genes were primarily selected based on their marked regulation in response to phorbol ester and their PKC isoform dependence in the LNCaP cells as described by Kazanietz and coworkers [34]. We posed the following questions. How did the compounds compare one to another in their effects on individual genes? Did each of the genes examined show a similar pattern of response to the three compounds? How did the patterns of response in the two cellular systems compare, given that Merle 23 appeared to act quite differently in the two systems? Additionally, we have previously described the degree to which a series of synthetic bryostatin derivatives behave like PMA or bryostatin 1 for cell attachment and inhibition of proliferation in the U937 cell system [35–38]. Here, we now compared their activities on several representative genes from the above analysis. The striking finding for these two cellular systems was that short duration of response was the predominant difference between bryostatin 1 and phorbol ester and correlated with the “bryostatin 1-likeness” of the various derivatives. Furthermore, the effect of Merle 23 was largely similar in the LNCaP and U937 cells, suggesting that the different biological outcomes in the two cell types largely depend on how the cells respond to the same modest mechanistic differences between PMA and Merle 23.

2. Methods

2.1. Materials

PMA and bryostatin 2 were purchased from LC Laboratories (Woburn, MA). Bryostatin 1 was provided by the Developmental Therapeutics Program, NCI (Frederick, MD). The syntheses of Merle 23, 28, 30, and 32 were described earlier [33, 35–37], as was the syntheses of the bryostatin analogue Merle 33 [Li, W., PhD Thesis, University of Utah, 2011]. The structures are illustrated in Figure 1. The LNCaP human prostate cancer cell line, the U937 human monocytic cell line, fetal bovine serum (FBS), and RPMI-1640 medium were obtained from ATCC (Manassas, VA). Precast 10% SDS gels, TNF α ELISA kit, SuperScript II Reverse Transcriptase, and PBS were from Invitrogen (Carlsbad, CA). The primary antibodies against PKC α (sc-208), PKC β _I (sc-209), PKC β _{II} (sc-210), PKC δ (sc-937), and PKC ϵ (sc-214) were from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibodies against the phosphorylated form of PKC δ (pPKC δ Y311), RasGRP3, TRAF1, pPKD1 (Ser744/Ser748), and PKD1 were from Cell Signaling (Danvers, MA), that against BIRC3 was from Epitomics (Burlingame, CA), and that against β -actin was from

Sigma (St. Louis, MO). The rabbit monoclonal antibody against the p-Ser299 of PKC δ was a custom antibody developed by Epitomics (Burlingame, CA). The iQ SYBR Green Supermix, the horseradish peroxidase conjugated secondary anti-rabbit antibodies, the non-fat dry milk, Tween-20, and the Triton X-100 solution were from Bio-Rad (Hercules, CA). The ECL (electrochemiluminescence) reagent and the films were from GE Healthcare (Piscataway, NJ).

2.2. Measurement of TNF α

Supernatants from LNCaP (300,000 cells/6 cm plate treated two days after plating) or U937 cells (4 ml of 150,000 cells/ml in 6 cm plates treated 24 hrs after plating) were collected 24 h after treatment, then centrifuged (4000 rpm for 5 min in an Eppendorf microcentrifuge) to remove any cells, and TNF α levels were measured by ELISA according to the manufacturer's (Invitrogen, Carlsbad, CA) instructions.

2.3 The growth and attachment of U937 cells

These cells were measured as described earlier [33]. U937 cells were plated in 35 mm dishes at a density of 1×10^5 living cells per mL, then treated 24 h later with different concentrations of the drugs dissolved in DMSO (DMSO concentration in each sample was 0.1 %). After 72 hrs, the number of unattached cells present in the supernatant and the number of attached cells (determined after trypsinization) were counted by using a Beckman particle counter (Beckman Coulter Inc., Fullerton, CA). The number of attached cells was expressed as the percentage of total cells.

2.4. Real time RT-PCR analysis

RNA was isolated from cultured cells with TRIzol reagent following the manufacturer's protocol (Invitrogen, Carlsbad, CA). For cDNA synthesis, 1.5 μ g of total RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Real time PCR was performed on MyiQ or iQ5 instruments (BIO-RAD, Hercules, CA) in a volume of 20 μ l using iQ SYBR Green Supermix from BIORAD (Hercules, CA) on 150 times diluted cDNA. The primers used were predesigned Quantitect primers from Qiagen (Valencia, CA) or, for GAPDH, PKC δ and RasGRP3, predesigned TissueScan primers from Origene (Rockville, MD). Relative gene expression levels were calculated using the $2^{-(\Delta\Delta C_t)}$ formula, where $\Delta\Delta C_t$ represents the cycle difference corrected for GAPDH, used as an internal control. The data are presented as fold change in gene expression normalized to GAPDH and relative to the DMSO treated control. The efficiency of the qPCR reactions was between 93% and 109 % when tested on serially diluted (1:5) "universal" RNA samples prepared to contain all transcripts of interest.

2.5. Western blot analysis

Total cell lysates, in ice cold phosphate buffered saline solution (PBS) containing 0.5% Triton X-100 and supplemented with protease and phosphatase inhibitors (Roche, Indianapolis, IN), were prepared by vortexing and sonication (3 times 6 sec). 20 μ g lysates (protein concentration measured using the BIO-RAD DC^{Tm} protein assay) were added to SDS and beta-mercaptoethanol containing sample buffer (Quality Biological Inc, Gaithersburg, MD), separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (Whatman GMBH, Germany). The membranes were blocked with 5 % nonfat dry milk, incubated overnight in the primary antibodies, washed (3 times 5 min in PBS with 0.05% Tween-20), incubated for 1 hr in the secondary antibody, and washed (3 times 5 min in PBS with 0.05% Tween-20). The signal was developed by ECL (Amersham, Piscataway, NJ) and detected on high performance chemiluminescence film (Amersham,

Piscataway, NJ). The scanned films were edited using Adobe Photoshop CS3 (Adobe Systems Inc).

2.6 Statistics

Changes in the levels of gene expression as determined by qPCR in response to PMA, bryostatin 1, and Merle 23 were expressed as Log2 values. The ratios relative to the level of expression in response to PMA were determined and the significance of the differences was calculated using a one sample t-test (hypothetical value of 1).

3. Results

Our previous comparison of the relative behavior of PMA, bryostatin 1, and Merle 23 in the LNCaP cells had yielded multiple mechanistic differences between the three compounds. Recently, Kazanietz and coworkers described the effect of phorbol ester treatment of the LNCaP cells on its pattern of global gene expression as determined by microarray [34]. That study further used siRNA treatment to explore the contribution of individual PKC isoforms to the gene expression pattern. Here, we have used a subset of the phorbol ester responsive genes reported in that study to compare the actions of PMA, bryostatin 1, and Merle 23 in the LNCaP cells. We also included in our analysis PKC δ and RasGRP3, two targets of phorbol ester action found in these cells. By limiting the number of genes examined, we were able to evaluate the dose response relations for each compound on each gene and to evaluate the time course of response for each compound at each dose for each gene. In our assays, the cells were exposed to the compound for the entire incubation time indicated. This protocol thus differs from that described in the study by Kazanietz and coworkers, in which cells were washed after 1 hr exposure to phorbol ester and incubation then continued in fresh medium [34].

For the LNCaP cells, we determined dose response curves for the 18 selected genes at 2, 6, 12, and 24 hr of treatment with PMA, bryostatin 1, and Merle 23, using 10-fold increments of the compounds and at least 2 independent experiments. Typical dose response curves for TNF α are illustrated in Figure 2 (Supplementary Figures 1A and 1B or data not shown for the other 17 genes). A possibility we had wished to examine was that different genes might be regulated through different targets (different PKC isoforms or other C1 domain containing proteins) with different selectivities for the three ligands. Although the ten-fold increments in compound concentrations in the dose response curves did not permit precise measures of EC₅₀ values, the genes showed approximately similar EC₅₀ values and the three compounds showed approximately similar potencies, although Merle 23 was up to 3–5-fold less potent in some cases. EC₅₀ values were generally between 1 and 10 nM, and concentrations between 10 and 1000 nM gave similar maximal responses, although the extents of the maximal responses were typically different for PMA, bryostatin 1, and Merle 23, depending on the gene and the time of treatment. (Figure 2, Supplementary Figure 1A and 1B, and data not shown). A common observation, as analyzed below, was that the curves for bryostatin 1 showed a diminished response with time relative to that for PMA, while the response to Merle 23 remained much closer to that for PMA.

The time courses for modulation of expression of all the genes at 100 nM PMA, a fully effective dose, are shown in Figure 3A. Levels of stimulation ranged up to 23,000-fold for the selected genes. Maximal response was predominantly at 6 hrs of incubation or later (12/17 responsive genes), but approximately a third of the genes fully responded by 2 hrs of incubation (5/17 responsive genes). The duration of response was variable. For many of the genes the response to PMA was largely maintained for 24 hrs (e.g. CXCL8, CCL2, TNF α , TRAF1, BIRC3). In some others it had largely returned to control levels (e.g. ALOXE3,

ETS2, SMAD6). While the expression of most genes was enhanced, levels of expression of SMAD6 and PPP1R3D were reduced.

We further analyzed the relative levels of expression in response to bryostatin 1 and Merle 23 of the genes for which PMA induced a change of expression of at least 2.5-fold the SD of the qPCR measurements ($n = 15, 15, 13$, and 12 genes at $2, 6, 12$, and 24 hrs, respectively). We compared the extent of response of 100 nM PMA to that of a fully effective dose of bryostatin 1 (100 nM) as a function of time (Figure 3B). The pattern of response to bryostatin 1 was striking and informative. First, we see that at 2 hr bryostatin 1 induced a generally similar extent of response to that of PMA, even though absolute levels of stimulation ranged up to four orders of magnitude. The average absolute change in gene expression in response to PMA at 2 hr was 26 -fold; the average bryostatin 1 response was 52% of the PMA response ($p = 0.0002$). By 6 hrs the extent of response to bryostatin 1 had become markedly less than that to PMA. The average changes in gene expression in response to PMA were 87 -, 71 -, and 83 -fold at $6, 12$, and 24 hrs; the corresponding average bryostatin 1 responses were 11% , 6.9% and 9.8% of the PMA response (p values all < 0.0003 for difference relative to PMA). The clear conclusion was that bryostatin 1 induced an overall similar pattern of gene expression to that induced by PMA. It differed, however, in that the induction by bryostatin 1 showed marked transiency, contrasting with the much more persistent response to PMA. It was also evident that the degree of transiency was not identical for all genes. In particular, SERPINB2 showed dramatic induction by bryostatin 1 at 2 hr which was entirely lost by 24 hr whereas the response to PMA was largely maintained.

We used a 1000 nM concentration of Merle 23, to again insure that we were using a fully effective dose for all genes. The pattern of response to Merle 23, like that to bryostatin 1, was also striking and informative (Figure 3B). Merle 23 induced essentially the same level of expression as did PMA at 2 hr, with an average change in response relative to that to PMA of 73% . At $6, 12$ and 24 hr the relative response to Merle 23 was reduced but not to the same degree as for bryostatin 1 (58% , 23% , and 35% , respectively; P values > 0.01). Again, SERPINB2 was an outlier, with Merle 23 causing much less induction at 24 hr than did PMA. We conclude that, in terms of its transcriptional effects in the LNCaP cells, Merle 23 was much more like PMA than like bryostatin 1. It showed a reduced duration of action compared to PMA but this reduction in duration was of appreciably lesser extent than was the case for bryostatin 1.

A trivial interpretation for transient response is that bryostatin 1 and, to a lesser degree, Merle 23 are simply unstable in the presence of cells. On the other hand, the consistent observation has been that, when bryostatin 1 failed to induce a typical phorbol ester response, bryostatin 1 suppressed the phorbol ester response when both compounds were administered together. We confirmed that this was the case in the LNCaP cells under the conditions of biological assays, where bryostatin 1 blocked the inhibition of proliferation induced by PMA [15]. In the current study, we likewise confirmed for a subset of the panel of the genes we examined that treatment with the combination of bryostatin 1 and PMA yielded levels of gene expression at 6 hrs corresponding to those for bryostatin 1 treatment alone rather than the much higher levels found upon treatment with PMA alone (Figure 4) (The average absolute change in gene expression in response to PMA for the subset of genes examined was 320 -fold, the average bryostatin 1 response was 4.1% , and the average bryostatin 1 plus PMA response was 7.4% of that of PMA ($p < 0.0001$)). Likewise, we showed that bryostatin 1 was able to suppress the higher level of gene expression observed at 6 hr in response to Merle 23 (the average response for bryostatin 1 plus Merle 23 was 11.2% of that of Merle 23 (270 -fold) ($p < 0.0001$)).

Although the focus of our analysis here is on patterns of gene expression in response to treatment with PMA, bryostatin 1, and Merle 23, we confirmed by immunoblotting (Figure 5) a similar pattern of PKC isoform down regulation and phosphorylation to that we had described before [15]. In contrast to the diminished effect of bryostatin 1 compared to PMA or Merle 23 on gene expression, we saw that PKC δ and PKC ϵ protein levels were maintained at 8 hr as was the phosphorylation of PKD1, a downstream substrate of PKC. We also saw the continued presence of PKC δ phosphorylated at S299, a phosphorylation site corresponding to the active enzyme [39]. BIRC3 and TRAF1 protein showed a much lower level of induction by bryostatin 1 compared to PMA and Merle 23, although the magnitude of the differences appeared somewhat less than seen at the level of mRNA expression. When cells were treated with the combination of bryostatin 1 together with either PMA or Merle 23, the responses resembled those for bryostatin 1, confirming that the dominant behavior of bryostatin 1 over PMA or Merle 23 effects could be observed at the protein level as well (Figure 5).

For biological response, Merle 23 behaved in an opposite fashion in the LNCaP cells and in the U937 cells. In the U937 cells Merle 23 was PMA-like, inhibiting cell proliferation and inducing attachment [33], whereas in the LNCaP cells it was bryostatin 1-like, not inhibiting proliferation or inducing apoptosis [15]. We therefore wished to compare in these two cells the patterns of gene expression to see how they related to the rather different patterns of biological response. Once again, we used qPCR to characterize gene expression in response to each compound as a function of dose and time. For our analysis, we tested the 18 genes analyzed in the LNCaP cells and we found significant changes in 8. Figure 6 summarizes response at 2, 8, and 24 hr to saturating doses of PMA, bryostatin 1, and Merle 23. In the U937 cells, the maximal levels of stimulation in response to PMA were not as high as seen in the LNCaP cells. For example, PMA treatment only elevated TNF α and CXCL8 38-fold (2 hrs) and 28-fold (24 hrs), respectively, as compared to the multiple thousand fold in the LNCaP cells (Figure 6A). In absolute terms, the action of bryostatin 1 showed less deviation from that of PMA as a function of time (Figure 6B). Bryostatin 1 induced a level of expression at 8 hrs only 2–3 fold less than that of PMA for most of the genes, although some, like TNF α or CXCL8, showed a larger difference. By 24 hrs, response was diminished in all cases for bryostatin 1 relative to PMA, although the differences were smaller than was found for the LNCaP cells. Consistent with the smaller difference in the U937 cells between the effects of PMA and bryostatin 1, Merle 23 likewise showed a smaller absolute difference with PMA (Figure 6B). In relative terms, however, the differences were somewhat greater. The average changes in gene expression in response to PMA were 4.8-, 9.1-, and 17-fold at 2, 8, and 24 hrs; the corresponding average bryostatin 1 responses were 75.4% ($p=0.0763$), 43.4% ($p=0.0151$) and 11.5% ($p<0.0001$) of the PMA response. The corresponding values for Merle 23 relative to PMA were 45.4%, 60.9% and 48.2%, respectively ($p<0.01$ for all values).

As with the LNCaP cells, we examined the ability of bryostatin 1 and Merle 23 to inhibit the PMA response in the U937 cells (Figure 7). Comparison was carried out at 24 hr after compound addition. In all cases in which bryostatin 1 induced a lesser response than did PMA, the combination was similar to that by bryostatin 1 alone (the average absolute change in gene expression for the subset of genes examined in response to PMA was 8.8-fold, the average bryostatin 1 response was 21.7 %, and the average bryostatin 1 plus PMA response was 20.0% of that of PMA)($p<0.0001$). Similarly, the combination of bryostatin 1 and Merle 23 gave the reduced level of expression seen for bryostatin 1 alone (the average response for bryostatin 1 plus Merle 23 was 24.1 % of that of Merle 23 (8.2 fold)($p<0.0001$).

Examination of the individual dose response curves for gene expression in response to the three compounds in the U937 cells yielded additional conclusions. First, in the U937 cells

the dose response curves for Merle 23 were shifted approximately one log to the right compared to bryostatin 1 (illustrated for CCL2 in Figure 8A). Second, the responses to bryostatin 1 at 8 or 24 hrs tended to be biphasic, with reduced response at the maximal dose tested (1000 nM) as compared to 1 nM. This was not the case for either PMA or Merle 23 (illustrated for CCL2 in Figure 8A; compiled data for all 8 genes illustrated in Figure 8B, other comparisons relative to the maximal dose, data not shown).

For comparison with the gene expression results, we characterized the down regulation and phosphorylation of PKC isoforms in the U937 in response to PMA, bryostatin 1 and Merle 23 (Figure 9). Bryostatin 1 was more effective than either PMA or Merle 23 at down regulation of PKC β_{II} and PKC β_I . PKC δ was only modestly reduced, and it remained phosphorylated at Ser299, as also seen in the LNCaP cells. In response to bryostatin 1 but not the other two agents, PKC δ showed a biphasic pattern of down regulation, with maximal reduction in response to 1 nM bryostatin 1. PKC ϵ was slightly downregulated by PMA and Merle 23 but unchanged by bryostatin 1 treatment. Bryostatin 1 induced lower levels of TRAF1 and RasGRP3 protein than did PMA or Merle 23. There was little difference in maximal BIRC3 protein levels, but the bryostatin 1 induced response was biphasic.

Analysis by the Wender group of the structure activity relations for bryostatin analogues had suggested the hypothesis that the A- and B-rings of the bryostatin functioned as a “spacer domain”, while protein kinase C interaction depended on proper orientation of residues in the lower half of the molecule [22]. Merle 23, which differs from bryostatin 1 only in lacking functionalities on these A- and B-rings, permitted evaluation of a somewhat different question, namely whether the unique pattern of biology which distinguishes bryostatin 1 from phorbol esters and other typical PKC ligands was also independent of the pattern of substitution on the A-and B-rings. The clear finding was that it is not, at least in the U937 cells [33]. Along with the more detailed mechanistic studies with Merle 23 [15], we have previously extended the structural analysis to probe the influence of different patterns of substitution of bryostatin derivatives on their activity in the U937 cells [35–37]. Here, we tested the series of bryostatin derivatives in the LNCaP cells for their induction of six of the phorbol ester regulated genes. All compounds were used at fully stimulatory doses, as evidenced by similar levels of gene expression at 2 hr (Supplementary Data, Figure 2). We find that the degree of response of the LNCaP cells at 8 hrs to the various derivatives ranged between that of PMA and that of bryostatin 1 or a little below (Figure 10A). Similar behavior was observed in the U937 cells that were treated for 24 hr (Figure 10B). Figure 10C shows the averaged gene expression data for all of the genes in each cell type, along with a summary of the extents to which the various derivatives resembled PMA or bryostatin 1 in their effects on U937 proliferation and attachment. We see strong correlation between the bryostatin-like patterns of gene expression and the bryostatin-like patterns of biological response in the U937 cells. The Spearman rank correlations between the extent of gene induction by the various compounds in the LNCaP cells and U937 growth inhibition and attachment were 0.881 and 0.986, respectively; the corresponding values for gene induction by the various compounds in the U937 cells and U937 cell growth inhibition and attachment were 0.69 and 0.83, respectively. We conclude that these patterns of gene expression provide a measure of how bryostatin 1-like a derivative may be.

Among the genes examined in this study, TNF α is of particular interest, in that the secretion of TNF α protein has been identified as an important contributor to the inhibition of LNCaP cell growth in response to phorbol ester [29–30]. We therefore examined, for the same series of bryostatin derivatives, the secretion of TNF α by the LNCaP cells (Figure 11A). Consistent with these derivatives being intermediate between Merle 23 and bryostatin 1 in their induction of TNF α mRNA expression at 8 hrs, the compounds proved to be intermediate in their levels of TNF α secretion and in all cases induced appreciably less

secreted TNF α than did PMA. It is worth noting, of course, that these assays of secreted TNF α are on a linear scale, reflecting the more limited dynamic range of these assays compared to the qPCR. We similarly examined the induction of TNF α secretion in the U937 cells (Figure 11B and Supplementary Figure 3). Similar general behavior was seen, but the magnitude of the differences was considerably smaller, again consistent with the lesser differences as revealed by qPCR.

4. Discussion

A difficulty in the understanding of bryostatin 1 action is that there are multiple mechanistic differences compared to the phorbol esters and it has been hard to assess the relative importance of these various mechanisms. While a short duration of response to bryostatin 1 followed by suppression has been one of the prominent differences in biology in various systems, differences in PKC δ localization, modification, and down regulation have provided competing alternatives. The present studies highlight a central role for transient duration of action of bryostatin 1 in its differential behavior compared to phorbol ester in the LNCaP cells. The close similarity in the levels of gene induction by PMA and bryostatin 1 at 2 hr in the LNCaP cells emphasizes that inducing different gene sets is not the predominant feature, at least for the series of genes examined. On the other hand, the relative action of bryostatin 1 rapidly diminishes thereafter, unlike in the case of PMA, and this diminution is the general observation over this gene set. A similar pattern was observed in the U937 cells, although the magnitude of the changes in gene expression in response to phorbol ester or bryostatin 1 was not as great and the response to bryostatin 1 did not diminish as quickly. The U937 cells thus argue that short duration of action as a central characteristic of bryostatin 1 action is not limited to the LNCaP cells.

Merle 23 represents a stringent test for the degree to which the A- and B-rings of bryostatin 1 constitute a “spacer domain” or, to the contrary, are a critical determinant of bryostatin-like activity. Both in the LNCaP cells and in the U937 cells, bryostatin 1 shows a transient duration of response compared to PMA. Although the action of Merle 23 is more transient than that of PMA in the LNCaP cells, the difference is limited compared to that of bryostatin 1. In the U937 cells the difference in response of Merle 23 relative to PMA is even less. We thus conclude that the transient duration of action of bryostatin 1 largely depends on the substituents on the A- and B-rings in both these two cell types. As we have described earlier [35–38], none of the substituents on these rings singly can account for the bryostatin 1 like behavior in the U937 cells. Rather, an appropriate combination is required. While overall lipophilicity may be important, it is by itself not determinative [40]. Finally, as illustrated both by the biology and the transiency of gene response, we can see that bryostatin 1 like is not an all-or-none property but rather a matter of degree.

The biological behavior of the bryostatin derivative Merle 23 had been puzzling in this regard, in that it resembled PMA in its effect on U937 cell growth and attachment whereas it resembled bryostatin 1 in its effect on growth and apoptosis in the LNCaP cells [15]. The present studies suggest that the difference in behavior of Merle 23 at the mechanistic level between these two cell types is actually rather small and in both cases mechanistically it largely resembles PMA. We think that the explanation is that the LNCaP cells are special in being poised on the margin of being growth inhibited, or not, by exposure to PKC ligands. This was seen in the diminution in phorbol ester effect at doses above the optimal dose (Figure 11A). It was likewise seen in the behavior of a number of phorbol esters which showed reduced induction of TNF α and reduced inhibition of LNCaP cell growth compared to PMA [40]. The differences of less than 4-fold in TNF α secretion in those studies with phorbol esters were largely mirrored in the differences in the extents of inhibition of LNCaP cell growth. In our present studies, although bryostatin 1 caused a 200-fold lower induction

of TNF α mRNA expression than did PMA, whereas Merle 23 only caused a 10-fold decrease, this 10-fold decrease in mRNA, which was associated with a 12-fold decrease in secreted TNF α (Figure 11A) would still be expected to be consequential. A related factor was the dramatic magnitude of the transcriptional responses to PMA in the LNCaP cells. A consequence is that a modest decrease in a very large response is still significant.

In contrast to the LNCaP cells, in the U937 cells the biological response to PMA cells appears more robust. Thus, the phorbol esters which showed diminished activity for biological response in the LNCaP cells caused responses very similar to those of PMA in the U937 cells [40]. Additionally, the magnitude of the stimulation of transcriptional responses to PMA was appreciably less and the reduction in response to bryostatin 1 was less. Thus, a compound like Merle 23, which in both systems was much more PMA-like than bryostatin-like at the transcriptional level, caused very little difference in response in secreted TNF α compared to PMA.

Comparing the LNCaP and U937 cells, we see that the extent of TNF α secretion is consistent with the biological behavior in response to PMA, bryostatin 1 and 2, and the various Merle compounds. In both cell types, there is a similar pattern of graded response of TNF α secretion for the various compounds, compared to their PMA- or bryostatin 1-like biological responses in the U937 cells. In the U937 cells, the lesser difference between PMA and Merle 23 for TNF α secretion fits with Merle 23 acting like PMA in this system. In contrast, in the LNCaP cells the greater absolute difference in secretion of TNF α , even though Merle 23 still induces appreciably more TNF α secretion than does bryostatin 1, fits with Merle 23 being bryostatin 1-like in this system. The transiency of the induction of TNF α provides at least one mechanism that would contribute to the differences in secretion. Biologically, in the LNCaP cells an important role for TNF α has been documented for the growth inhibitory effects of the phorbol esters [29–30], as is also the case for the U937 cells [41]. On the other hand, the other genes we examined for response to the various derivatives behaved similarly to TNF α , so their contributions as well as those of all the genes we did not examine should not be discounted.

In interpreting the structure activity relations, it should be noted that the modest extent of transient duration of response seen in the LNCaP cells in response to Merle 23 may not have the same origin as that in response to bryostatin 1. As we have described earlier, in the LNCaP cells the level of PKC δ in the nuclear fraction correlated with responsiveness of the LNCaP cells [15]. In the case of bryostatin 1, the localization to the nuclear fraction was lost in the face of persistent total PKC δ levels. In the case of Merle 23, it was the total levels of PKC δ which declined with a corresponding decrease in PKC δ in the nuclear fraction. The treatment with the proteasome inhibitors reversed this latter effect but not the former, corresponding to the shift in biological action of Merle 23 but not bryostatin 1 to being PMA-like.

An on-going obstacle in the evaluation of bryostatin structure activity relations has been limited material. Our findings suggest that duration of response, as evidenced by changes in levels of mRNA induction, should provide a convenient and powerful tool for initial evaluation of the “bryostatin likeness” of bryostatin derivatives. In addition to being compound sparing, a further advantage of this approach is that the duration of expression was not very sensitive to dose once saturating concentrations of ligand have been achieved and that full effect at early times could be used to confirm that the dose was saturating. The strategy measuring duration of response should thus be suited to structure activity analysis using combinatorial chemistry, where compound quantity and purity may not be tightly controlled.

Although the focus of this study has been on transient duration of action, several other features are noteworthy. RasGRP3 was dramatically up-regulated (>64-fold) in the U937 cells in response to PMA but not bryostatin 1. This level of induction was the greatest in any of the 8 genes examined and, furthermore, the induction was confirmed upon immunoblotting. Since RasGRP3 is also a phorbol ester responsive gene, it undoubtedly will contribute to the pattern of response of the U937 cells at later times of PMA exposure. Previously, RasGRP3 has been described as being up-regulated in human vascular endothelial cells during angiogenesis, although not to the same extent [42].

It is also important to emphasize that, more generally, mechanisms other than transient duration of action must contribute to the differential activity of bryostatin 1 and PMA. We clearly showed in the HOP92 lung cancer cell line that the pattern of PKC δ down regulation and, at higher bryostatin 1 doses, protection from down regulation were directly reflected in the difference in growth response of this cell type [43]. In the C3H10T1/2 cells, bryostatin 1 was deficient in causing release of arachidonic acid metabolites even at early times although its inhibition of EGF binding was of long duration [18]. The degree to which our conclusions from study of the U937 and LNCaP cells apply to other cell types remains the subject of future investigation. Adequate understanding the relative contributions of different mechanisms in different cell types will be critical for identifying those cancers in which bryostatin 1, as a PKC antagonist, has rational application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structure of PMA, bryostatin 1, Merle 23, and the various derivatives.

Figure 2. Time and dose dependent changes in mRNA levels in LNCaP cells

Real time RT-PCR analysis of TNF α RNA prepared from LNCaP cells treated with the indicated drug concentrations for 2, 6, or 24 hours. Average values $\pm$ SEM from three independent experiments are shown.

Figure 3A

Figure 3B

Figure 3. Time dependence of the modulation of mRNA levels of specific genes in LNCaP cells
 Real time PCR analysis of 18 genes. **A)** Relative levels of the mRNA upon treatment with 100 nM PMA for 2, 6, 12 or 24 hours (columns from left to right) compared to the DMSO control. **B)** Relative levels of the mRNA upon treatment with 100 nM PMA, 100 nM bryostatin 1 or 1000 nM Merle 23 for 2, 6, 12 or 24 hours compared to the DMSO control. Data represent average $\pm$ SEM values from at least two independent experiments ($n = 2-4$).

Figure 4. The effect of bryostatin 1 on gene induction is dominant over that of PMA or Merle 23 in LNCaP cells

Real time RT PCR analysis was performed for the indicated genes on RNA prepared from cells treated for 6 hours as indicated by the shaded squares with 100 nM PMA, 100 nM bryostatin 1, 1000 nM Merle 23 or their combinations. Data represent the average $\pm$ SEM from two independent experiments.

Figure 5. Changes at the protein level induced by PMA, bryostatin 1 or Merle 23 in LNCaP cells
Immunoblotting of total cell lysates prepared from LNCaP cells treated for 8 hours with the indicated concentrations of PMA, bryostatin 1, or Merle 23 or their combinations. Representative images from three independent experiments are shown.

Figure 6A

Figure 6B

Figure 6. Time dependence of the modulation of mRNA levels of specific genes in U937 cells
 Real time RT-PCR analysis of 8 genes. **A)** Relative levels of mRNA upon treatment with PMA for 2, 8, or 24 hours (columns from left to right) compared to the DMSO control. **B)** Relative levels of the mRNA upon treatment with 100 nM PMA, 100 nM bryostatin 1 or 1000 nM Merle 23 for 2, 8 or 24 hours compared to the DMSO control. Data represent average $\pm$ SEM values from two (2 and 24 hrs) or three (8 hrs) independent experiments.

Figure 7. The effect of bryostatin 1 on gene induction is dominant over that of PMA or Merle 23 in U937 cells

Real time RT PCR analysis was performed for the indicated genes on RNA prepared from cells treated for 24 hours as indicated by the shaded squares with 100 nM PMA, 100 nM bryostatin 1, 1000 nM Merle 23 or their combinations. Data represent average $\pm$ SEM values from two independent experiments.

Figure 8A

Figure 8B

Figure 8. Time and dose dependent changes in mRNA levels in U937 cells

A) Real time RT-PCR analysis of CCL2 was performed on RNA prepared from cells treated with the indicated drug concentrations for 2, 8, or 24 hours. Average values from two (2 hrs) or three (8 and 24 hrs) independent experiments are shown. **B)** Ratios of gene induction by 1 nM vs 1000 nM PMA, by 1 nM vs 1000 nM bryostatin 1, and by 10 nM versus 1000 nM Merle 23 are plotted for the 8 genes examined in Figure 5 at 8 hrs. Values greater than 1 are indicative of a biphasic dose response curve. Data represent the average values of two independent experiments and the lines represent the geometric means of data points. $p = 0.0019$, Kruskal-Wallis test, for difference in ratio for bryostatin 1.

Figure 9. Changes at the protein level induced by PMA, bryostatin 1 or Merle 23 in U937 cells
 Immunoblotting of total cell lysates prepared from U937 cells treated for 24 hours with the indicated concentrations of PMA, bryostatin 1, or Merle 23 or their combinations. Representative images from three independent experiments are shown.

Figure 10A

Figure 10B

Figure 10C

Figure 10. Transcriptional activity of different bryostatin analogues in LNCaP cells at 8 hrs
A) Real time RT-PCR analysis was performed for the indicated genes on RNA prepared from LNCaP cells treated for 8 hours with 1000 nM of the indicated compounds. Data represent average $\pm$ SEM values from two independent experiments. **B)** Real time RT-PCR analysis was performed for the indicated genes on RNA prepared from U937 cells treated for 24 hours with 1000 nM of the indicated compounds (100 nM in the case of bryostatin 2). Average $\pm$ SEM values from three independent experiments are shown. **C)** The averaged effect of bryostatin analogues on gene expression in LNCaP and U937 cells and their biological effects in U937 cells. The two upper panels represent the mean $\pm$ SEM of the log values of changes described for panels **A** and **B**. The two lower panels represent the biological changes of inhibition of cell proliferation and induction of cell attachment in U937 after 72 hours of treatment with 1000 nM of the indicated compounds. Data represent mean $\pm$ SEM of three or more independent experiments. M23, M32, M33, M30, M28 are abbreviations for corresponding Merle compounds, and bryo 2 or Br 2, and bryo 1 or Br1 indicate bryostatin 2 and bryostatin 1, respectively. The biological data for M23, M30, M28, and M32 are from our previous reports [33, 35–37]. The values for PMA and Br1 represent the average from those reports together with additional experiments.

Figure 11A

Figure 11B

Figure 11. TNFα secretion induced by different bryostatins derivatives

A) TNFα concentrations were measured by ELISA in the supernatants of LNCaP cells treated for 24 hr with different doses of the indicated compounds (1, 10, 100, 1000 nM, respectively, from left to right). Data represent average $\pm$ SEM of two independent experiments (missing bars, not detectable). **B)** TNFα concentration was measured by ELISA in the supernatants of U937 cells treated for 24 hrs with 1000 nM of the indicated

compounds (100 nM for bryostatin 2). Data represent the average $\pm$ SEM of three independent experiments. M23, M32, M33, M30, M28 are abbreviations for the corresponding Merle compounds, and Br 2, and Br1 indicate bryostatin 2 and bryostatin 1, respectively.

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