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The Biological Profile of the Less Lipophilic and Synthetically More Accessible Bryostatin 7 Closely Resembles That of Bryostatin 1

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Abstract

The bryostatins are a group of twenty macrolides isolated by Pettit and coworkers from the marine organism *Bugula neritina*. Bryostatin 1, the flagship member of the family, has been the subject of intense chemical and biological investigations due to its remarkably diverse biological activities, including promising indications as therapy for cancer, Alzheimer's disease, and HIV. Other bryostatins, however, have attracted far less attention, most probably due to their relatively low natural abundance and associated scarcity of supply. Among all macrolides in this family, bryostatin 7 is biologically the most potent PKC (protein kinase C) ligand (in terms of binding affinity) and also the first bryostatin to be synthesized in the laboratory. Nonetheless, almost no biological studies have been carried out on this agent. We describe herein the total synthesis of bryostatin 7 based on our pyran annulation technology, which allows for the first detailed biological characterizations of bryostatin 7 with side-by-side comparisons to bryostatin 1. The results suggest that the more easily synthesized and less lipophilic bryostatin 7 may be an effective surrogate for bryostatin 1.

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Supporting Information

Chemical procedures and spectral data for synthesized compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Protein kinase C (PKC) is a group of serine/threonine kinases that play a critical role in signal transduction events that regulate cellular processes such as proliferation, differentiation, motility, inflammation, and apoptosis.(1) Inhibition or activation of PKCs has been linked to several human disease states including cancer,(2, 3, 4) Alzheimer's disease,(5) diabetes,(6) acquired immune deficiency syndrome (AIDS) (7) and cardiovascular diseases.(8) Thus, PKC has emerged as an attractive target for drug discovery and several natural products and synthetic molecules have been identified as modulators of PKCs.(9, 10, 11).

The phorbol esters and indolactams are some of the most studied PKC activators and are well-known to bind to the C1 domain of PKCs with high affinity.(12, 13) Although the phorbol esters provided a paradigm for tumor promoters,(14) suggesting that activation of PKC should thus be tumor promoting, it has since become clear that the situation is much more complex. For example, the structurally similar phorbol esters PMA (**1**) and prostratin (**2**) both activate PKC, but PMA is a tumor promoter whereas prostratin inhibits tumor promotion (Figure 1).(15, 16) The difference in their PKC binding affinities and activity in living cells can be best explained by the presence of a very lipophilic side chain at the C12 position of PMA. The more lipophilic PMA has a higher affinity for PKC and is a tumor promoter whereas the less lipophilic prostratin is not. Replacement of the 13-acetate of prostratin with 13-tetradecanoate likewise restores tumor promoting activity.(17) A similar trend in lipophilicity versus binding affinity is observed with indolactam V (**3**) and its more lipophilic analogue (**4**), where the latter has binding affinity about twenty times higher than that of the parent compound.(18) However, in this case both compounds are tumor promoters. From these examples, it is clear that there exists at least some correlation between lipophilicity and biological activity of the PKC ligands. Among biochemical correlations, the lipophilicity of ligands influences the pattern of PKC delta localization that they induce. We have shown that, for a series of known PKC activators of varying lipophilicity, the most lipophilic of these translocate GFP labeled PKCdelta exclusively to the plasma membrane, in marked contrast to the patterns of translocation observed with the less lipophilic agents, which favor translocation to the nuclear membrane and internal membranes.(19,20,21)

The bryostatins are another important class of antitumor natural products that bind to the C1 domain of PKC with exceptionally high affinity.(22) Among them, bryostatin 1 (**5**) is the most thoroughly studied member and has been subjected to numerous Phase I and II clinical trials for cancer chemotherapy.(23) It has been shown to reverse multidrug resistance in cancer cells,(24) synergize with other oncolytic agents, (25, 26, 27) and stimulate the immune system. (28, 29). Additionally, bryostatin 1 has exhibited promising neurological activity by improving learning and memory in animal models (30, 31, 32) and by reducing the formation of amyloid plaques in transgenic mice.³ As a result, a clinical trial for the treatment of Alzheimer's disease has been scheduled.(33) Recently, bryostatin 1 has also been shown to activate latent HIV infection in lymphocytes,(34) which is a promising lead indication for the use of this agent in HIV therapy. Although bryostatin 1 is the most abundant of the bryostatins, difficulties of supply are still a major impediment.

Despite immense interest in bryostatin 1 and its analogues, very little is known about the biological profiles of other naturally occurring bryostatins, most probably due to the scarcity of material for biological testing. Among other bryostatins, bryostatin 7 (**6**) deserves special mention because this congener was reported to have the highest binding affinity towards PKC (Figure 1).(35) Bryostatin 7 was isolated by Pettit and coworkers in 1984 from *Bugula neritina* collected from the Gulf of Mexico.(36) Ecologically; it is interesting that *Bugula neritina* collected from this region had no trace of bryostatin 1 whereas those from Gulf of

California contained mostly bryostatin 1. Despite the promising initial biological activity of bryostatin 7, almost no further investigation has been reported. In a single study reported by Wallace and coworkers, it has been suggested that bryostatin 7 mimics the activity of the phorbol ester PMA in inducing aggregation of human platelets by directly activating PKC, (37) a response also induced by bryostatin 1.(38) The studies described herein were conducted to define a detailed biological profile for bryostatin 7 and to determine the extent to which the activity of this more easily synthesized material resembled that of bryostatin 1.

RESULTS AND DISCUSSION

Synthesis of Bryostatin 7

We sought to characterize the biological profile of bryostatin 7 by thoroughly comparing its activity with the structurally related antitumor agent bryostatin 1 and also with the tumor promoting phorbol ester PMA. This required the preparation of bryostatin 7 by chemical synthesis (see supporting information) as this agent is not available by other means. This could be accomplished from a late intermediate in our previously published synthesis of bryostatin 1. (39) Briefly, the total synthesis of bryostatin 7 commenced from the fragment coupling of the A-ring hydroxy allylsilane **7** and the C-ring aldehyde **8** via pyran annulation to form the tricyclic intermediate **9** in Scheme (1).(40) Functional group manipulations on intermediate **9** according to previously reported methods provided the macrocycle **10** which has the fully functionalized and protected bryostatin 7 structure. A global deprotection of intermediate **10** with LiBF₄ in acetonitrile at 80 °C removed four protecting groups, *i. e.* one triethylsilyl, one benzyloxymethyl, and two methylketals to provide bryostatin 7 in 80% yield.(41) We were pleased to find that the C20 acetate could be selectively removed in the presence of the C7 acetate, the macrocyclic ester, and two methyl esters by using K₂CO₃ in methanol for 45 minutes. However, if the reaction was allowed to proceed longer, the C7 acetate could also be removed. This suggests that selective removal of a C7 acetate could also be possible provided a bulkier ester was present at C20. This result is significant for bryostatin syntheses since most of the members differ in substitutions at the C7 and C20 positions. Thus, using sequential acetate removal and esterification, bryostatins with varying substituents at these two positions should be accessible from the common intermediate **10**. As an example, a selective methanolysis of the C20 acetate followed by esterification of the resulting alcohol with (2*E*,4*E*)-octa-2,4-dienoic anhydride and a global deprotection provided bryostatin 1.

Determination of Binding Affinity

The biological evaluation of bryostatin 7 began by measuring its binding affinity towards purified PKC α . Bryostatin 7 bound to mouse PKC α with a K_i of 0.26 $\pm$ 0.06 nM compared to a K_i of 0.48 $\pm$ 0.03 nM for bryostatin 1, assayed under the same conditions (Table 1). This result is of interest since the less lipophilic bryostatin 7 binds slightly better than bryostatin 1 whereas the opposite trend was observed with other PKC ligands such as phorbol esters and indolactams.

To examine possible PKC isoform selectivity *in vitro*, we also determined the binding affinities of bryostatin 7 for human PKC isoforms α , β II, δ , and ϵ (Table 1). PKC β II, δ , and ϵ are the predominant phorbol ester sensitive PKC isoforms in the U937 leukemic cell line and PKC α , δ , and ϵ are the predominant phorbol ester sensitive PKC isoforms in the LNCaP prostate cancer cell line.(42) Both lines respond differentially to phorbol ester and bryostatin 1, and we have used them extensively to explore the structure activity relations of bryostatin derivatives that confer this differential behavior.(43) Under our *in vitro* binding conditions, bryostatin 7 showed little selectivity among these PKC isoforms, binding modestly more strongly than did bryostatin 1. The phorbol ester PDBu likewise showed

little selectivity under these conditions, as previously reported.(44) Thus for any of the three agents, binding affinities for the different isozymes were very similar, and the binding affinities of the three agents for any particular isozyme were also very similar. The observed differences are far too small to account for any significant differences in biology.

Biological Responses in U937 Leukemia Cells

The U937 human leukemia cell line is a well characterized system in which phorbol esters induce a differentiation response, as manifested by growth inhibition and cellular attachment, whereas bryostatin 1 induces a markedly reduced response.(45) If both bryostatin 1 and phorbol ester are co-administered, the response resembles that of bryostatin 1 alone, showing that the action of bryostatin 1 is dominant over that of phorbol ester. We have previously described the critical role played by the pattern of substitution of the A- and B-rings of bryostatin analogs in determining this bryostatin response. We show here that bryostatin 7 closely resembled bryostatin 1 in its effects on the U937 cells. Like bryostatin 1, bryostatin 7 caused a very limited, biphasic inhibition of U937 cell proliferation (Figure 1A). Similarly, like bryostatin 1, bryostatin 7 was able to suppress the growth inhibition induced by PMA. Once again, like bryostatin 1, bryostatin 7 caused a much reduced level of cell attachment compared to that induced by PMA and, when co-administered, was able to inhibit the attachment induced by PMA (Figure 1B). For both responses, the dose response curve for bryostatin 7 in these cells was shifted approximately 3-fold to higher concentrations compared to that for bryostatin 1. Also, although the differences were minor, a comparison of the dose response curves indicated that bryostatin 7 inhibited proliferation slightly more than did bryostatin 1 ($p = 0.002$, paired t-test) and induced slightly more attachment than did bryostatin 1 ($p = 0.008$, paired t-test). Finally, in the U937 cells we compared the secretion of tumor necrosis factor alpha (TNF α) in response to PMA, bryostatin 1, and bryostatin 7. TNF α has been suggested as an important contributor to the induction of apoptosis in U937 cells by PMA.(46) Bryostatin 1 and bryostatin 7 induced similar levels of TNF α secretion with similar potencies, whereas PMA induced a higher level of secretion (Figure 1C).

Bryostatin 7, like bryostatin 1, synergizes with araC to induce loss of mitochondrial potential in U937 cells

One of the therapeutic approaches for use of bryostatin 1 in cancer therapy is to use it in combination with other agents.(25–27) For example, bryostatin 1 has been reported to sensitize the U937 cells to toxicity by araC, measured by loss of mitochondrial membrane potential.(47) We confirm that bryostatin 7, like bryostatin 1, was able to synergize with araC in this assay (Figure 2D).

Down regulation of PKC isoforms in U937 cells

In the U937 cells, the prominent PKC isoforms are PKC β II, PKC δ , and PKC ϵ . Bryostatin 1 and 7 induced similar levels of down regulation of PKC β II, whereas PMA treatment induced less down regulation (Figure 3A). The potency of bryostatin 7 was 3-fold weaker than that for bryostatin 1 ($EC_{50} = 0.97 \pm 0.08$ nM versus 0.310 ± 0.005 nM), similar to the difference in potencies seen in the assays of cell proliferation and attachment. The modest increase in the PKC β II signal at high concentrations of bryostatin 1 or 7 reflected accumulation of a higher mobility form (only faintly visible at this exposure), which represents the unphosphorylated form of PKC β II.(48) For PKC δ , bryostatin 1 induced down regulation with a biphasic dose response curve, as observed in other cellular systems, whereas PMA caused little down regulation (Figure 2B). Once again, bryostatin 7 resembled bryostatin 1 but showed approximately 3-fold weaker potency ($EC_{50} = 0.22 \pm 0.03$ nM versus 0.083 ± 0.008 nM), indicating that bryostatin 7 and bryostatin 1 showed similar

selectivity between PKC β II and PKC δ . PKC ϵ showed little down regulation by either PMA or bryostatin 1 in these cells (data not shown) and was not examined here.

Biological responses in LNCaP human prostate cancer cells

The LNCaP prostate cancer cell line is a second well characterized system which responds differentially to phorbol ester and to bryostatin 1. PMA inhibits cellular proliferation; bryostatin 1 itself shows little inhibitory effect but, if co-administered with PMA, prevents the inhibition by PMA. Induction of tumor necrosis factor α (TNF α) by PMA is thought to be a major mediator of this growth inhibition(46); correspondingly, induction of TNF α by bryostatin 1 is much reduced.(43) Like bryostatin 1 and unlike PMA, bryostatin 7 fails to inhibit proliferation of the LNCaP cells (Figure 4A). Again, like bryostatin 1, bryostatin 7 is able to block the inhibition of proliferation by PMA (Figure 4B). Like bryostatin 1 and unlike PMA, bryostatin 7 causes minimal secretion of TNF α (Figure 4C). Like bryostatin 1, bryostatin 7 inhibits the secretion of TNF α induced by PMA (Figure 4D).

Translocation of GFP-PKC δ in LNCaP cells

We have previously described that bryostatin 1 differed from PMA in the pattern of membrane translocation of PKC δ that it induced.(19) Using mouse PKC δ tagged with GFP (green fluorescent protein) and transiently transduced into CHO (Chinese hamster ovary) cells, we had found that bryostatin 1 induced translocation of mouse GFP-PKC δ to the nuclear membrane and internal membranes, whereas PMA initially induced translocation to the plasma membrane, with subsequent partial redistribution to the nuclear membrane and internal membranes. Comparison of a range of phorbol esters and related ligands that differed in their tumor promoting abilities suggested that these different patterns of PKC δ translocation correlated with the tumor promoting ability of the compounds; the compounds that were tumor promoting induced a pattern resembling that induced by PMA, whereas those that were either non-promoting or inhibited tumor promotion gave patterns that resembled the pattern induced by bryostatin 1.(19,21) Since higher lipophilicity was associated with the tumor promoting compounds like PMA, a prediction is that bryostatin 7, which is less lipophilic than bryostatin 1, might induce even less plasma membrane translocation of GFP-PKC δ than did bryostatin 1. To test this prediction, we transiently transfected LNCaP cells with mouse GFP-PKC δ and monitored the response to bryostatin 1, bryostatin 7, and PMA. The LNCaP cells, like the U937 cells, are a cellular system in which the differential actions of phorbol esters and bryostatin have been studied extensively and, because they spread well on the substratum, are well suited for visualization of PKC translocation.(43,49) As predicted, bryostatin 7, like bryostatin 1, caused translocation of mouse GFP-PKC δ only to the nuclear membrane and internal membranes, whereas PMA caused initial translocation to the plasma membrane (Figure 5A). Using transiently transfected human PKC δ rather than mouse PKC δ , we saw some initial plasma membrane translocation in response to bryostatin 1, although it was still less prominent than that in response to PMA. In contrast, bryostatin 7 still only caused translocation of the human GFP-PKC δ to nuclear and internal membranes (Figure 5B), consistent with its lower lipophilicity. The images shown are representative of the multiple experiments performed (see figure legend for number of replicates under each condition) and thus reflect a general response rather than being limited to a minor population of the cells.

Translocation of YFP-PKC ϵ in LNCaP cells

Because of the difference in the pattern of translocation of PKC δ in response to bryostatin 1 and bryostatin 7, we further compared the response of human YFP-PKC ϵ to treatment with PMA, bryostatin 1, and bryostatin 7. The difference was dramatic. PMA caused rapid clearing of the cytoplasm with translocation of PKC ϵ to the plasma membrane. Translocation was a little faster in the case of the mouse PKC ϵ (data not shown) and a little

slower in the case of the human PKC ϵ (Figure 6). Bryostatin 1 treatment resembled that with PMA, albeit less extensive. Bryostatin 7, in contrast, caused no plasma membrane translocation, with shift to an internal punctate distribution consistent with association with internal membranes. The images shown are representative of the multiple experiments performed (see figure legend for number of replicates under each condition) and thus reflect a general response rather than being limited to a minor population of the cells. In light of the important roles ascribed to PKC ϵ in cancer and dementia, this differential activity by bryostatin 7 is particularly exciting.

Translocation of GFP-PKC α in Chinese Hamster Ovary (CHO) cells

Unlike the novel PKC isoforms PCK δ and PKC ϵ , PKC α translocation depends both on ligand interaction at the C1 domain and on intracellular calcium binding to the C2 domain. In the LNCaP cells, translocation of GFP-PKC α , both human and mouse, was slower with PMA and still slower with bryostatin 1 (data not shown); visualization was therefore complicated because of the changes in cell shape which develop in this cell type with time after ligand addition. As an alternative, we used expression of the GFP-PKC α in Chinese Hamster (CHO) cells to obtain clearer comparisons. The CHO cell system has been commonly used to visualize GFP-PKC translocation in response to various treatments. (19,21) PMA caused slow but clear plasma membrane translocation of human GFP-PKC α (Figure 7). In contrast, bryostatin 1 induced a more complicated pattern, with some translocation of the human PKC α to the plasma membrane but with most forming a thick ring around the nucleus (Figure 7). The images shown are representative of the multiple experiments performed (see figure legend for number of replicates under each condition) and thus reflect a general response rather than being limited to a minor population of the cells. This difference in the pattern of PKC α translocation in response to PMA and bryostatin 1 has been described previously for the IEC-18 rat intestinal epithelial cell line. (50) Bryostatin 7 induced a pattern of translocation similar to that of bryostatin 1. Results with mouse GFP-PKC α were similar to those for the human GFP-PKC α (data not shown).

Change in subcellular distribution of PKC isoforms in LNCaP cells upon ligand treatment as determined by cellular fractionation

A complementary approach for examining the effect of ligands on distribution of PKC is subcellular fractionation. Given the multiple changes in PKC microenvironment that might occur during the fractionation, it is probably less predictive of the actual distribution of PKC in the native cells but should still help identify differences. We compared the response to PMA, bryostatin 1, and bryostatin 7 of PKCs in the cytoplasmic and nuclear enriched fractions (Figure 8A). Bryostatin 7 resembled bryostatin 1 in its effects, whereas PMA showed a clearly distinct pattern. PKC α and PKC ϵ showed a similar shift to the nuclear enriched fraction in response to all three ligands. An increase in PKC δ in the nuclear enriched fraction was only observed for PMA. This difference between PMA and bryostatin 1 in accumulation of PKC δ in the nuclear enriched fraction had been reported by us previously(43); bryostatin 7 resembled bryostatin 1 for this response. pPKD1 largely resembled PKC δ in that only PMA caused substantial accumulation in the nuclear fraction. Once again, we had described previously this result for PMA and bryostatin 1.(43) We confirmed that finding here and showed that bryostatin 7 behaved similarly to bryostatin 1. The reduced presence of the pPKD1 in the nuclear enriched fraction reflected a combination of reduced PKD1 in the nuclear enriched fraction along with a reduced level of its phosphorylation.

The above fractionation conditions had been optimized for evaluating the nuclear enriched fraction, which we had previously described as showing clear differences in response to PMA and bryostatin 1.(43) To better define depletion of PKC isoforms from the cytoplasm,

we also prepared cytosolic and particulate fractions under conditions that should include all membranes in the particulate fraction.(51) All three ligands, PMA, bryostatin 1, and bryostatin 7 caused similar depletion of PKC α , PKC δ , and PKC ϵ from the cytosolic fraction (Figure 9), once again arguing that the action of the ligands was not limited to a subpopulation of the cells.

Comparison of the effect of ligands on PKC and downstream signaling in LNCaP cells

To further compare the actions of bryostatin 7 with bryostatin 1 in the LNCaP cells, we analyzed the above cells treated with PMA, bryostatin 1, or bryostatin 7 for responses downstream of PKC activation (Figure 8B). Bryostatin 7 and bryostatin 1 were closely similar for induction of PKC δ phosphorylation at S299, a measure of activated PKC δ , and for their inability to induce efficient PKC δ phosphorylation on Y311. Bryostatin 7 was likewise similar to bryostatin 1 in its stimulation of Erk phosphorylation. On the other hand, bryostatin 7 caused reduced induction of cFos or EGR1 at 60 min compared to bryostatin 1. Additionally, bryostatin 7 caused somewhat less phosphorylation of PKD1 than did bryostatin 1 (Figure 8A).

Gene induction in LNCaP cells

PMA induces marked changes in gene transcription in LNCaP cells.(52) For a series of phorbol ester responsive genes, we compared the effect of bryostatin 1 and bryostatin 7 with that of PMA (Figure 10). We observed that the changes in levels of mRNA expression were generally similar for bryostatin 1 and bryostatin 7 and less than they were for PMA. This difference was greater at 6 hours than at 3 hours of treatment (data not shown), consistent with our previous observation that bryostatin 1 displayed a more transient time course of action in these cells for a number of responses.(43)

Conclusions

We conclude that bryostatin 7 retains an overall pattern of biological response very similar to that of bryostatin 1. This finding is important, because the C20 acetate substituent on bryostatin 7 is both synthetically more convenient to install and also renders the molecule less lipophilic. This decrease in lipophilicity is reflected in the pattern of PKC δ translocation which it induced, where there was even less plasma membrane translocation than seen with bryostatin 1. The selectivity between PKC β II and PKC δ and the extents of PKC isoform down regulation and the changes in gene transcription were very similar between bryostatin 7 and bryostatin 1. Among modest differences were 3 fold lower potency of bryostatin 7 in the U937 cells, a slightly elevated level of cellular attachment, and a slightly greater degree of inhibition of proliferation. The different pattern of translocation of PKC ϵ in response to bryostatin 7, compared to either PMA or bryostatin 1, suggests that it may have some additional selectivity. Given the difficulties in formulating the lipophilic compound bryostatin 1 for IV infusion in clinical trials, the lower lipophilicity of bryostatin 7 may be advantageous. Moreover, these results very clearly show that the long C20 sidechain is not a critical structural element and is not necessary to obtain the biological responses characteristic of bryostatin 1.

METHODS

Materials

PMA was purchased from LC Laboratories. Bryostatin 1 was provided by the Developmental Therapeutics Program, NCI. 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. Precast 10% SDS gels, TNF α ELISA kit, SuperScript II Reverse

Transcriptase, PBS, MitoTracker Red CMXRos, and the human PKC isoforms were from Invitrogen. The primary antibodies against PKC β II (sc-210), PKC δ (sc-937), PKC ϵ (sc-) and cFos (sc-7202) were from Santa Cruz Biotechnology, the ones against PKC α (C-terminal), pPKC δ S299, pPKC δ Y311, and EGR1 were from Epitomics. The primary antibody against β -actin was from Sigma and the ones against α -tubulin, PKD1, pPKD1S744S748, pERK, ERK, Lamin B1 and TATA binding protein were from Cell Signaling Technology. 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. The ECL (electrochemiluminescence) reagent and the films were from GE Healthcare.

The growth and attachment of U937 cells

Cells were measured as described earlier.(53) 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 60 h, 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. The number of attached cells was expressed as the percentage of total cells.

The growth of LNCaP cells was determined using an Incucyte instrument as described earlier.(43)

Measurement of TNF-alpha

Supernatants from U937 cells (4 ml of 150,000 cells/ml in 6 cm plates treated 24 h after plating) and LNCaP cells (120,000 cells/ml in 24 well plates treated 24 h 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) instructions.

Simple Western™

U937 cells treated with DMSO or concentrations of PMA, byrostatin 1, or bryostatin 7 as indicated were lysed with M-PER (Thermo Scientific) buffer containing phosphatase and protease inhibitors (EMD Millipore). Lysates were mixed with a master mix containing DTT, sample buffer, and molecular weight fluorescent standards and then boiled at 95 °C for 5 minutes. Samples and reagents were loaded onto a 384 well plate for analysis by the Simple Western System, an automated western performed in capillaries and developed by ProteinSimple.(54) In this study, 10–24 ng of lysate was loaded into each capillary and proteins were separated by molecular weight as they moved through stacking and separation matrices for 40 minutes at 250 V. Proteins were immobilized to capillary walls using proprietary, photoactivated capture chemistry. Capillaries were then incubated with a blocking reagent and target proteins were immunoprobed with anti-PKC δ or anti-PKC β II and either anti- β -actin or anti- α -tubulin primary antibodies (Sigma Aldrich and Cell Signaling Technology, respectively) and horseradish peroxidase-conjugated anti-mouse and/or anti-rabbit secondary antibodies (Jackson ImmunoResearch). A mixture of luminol and peroxide was added, the resulting chemiluminescent signal was captured by a CCD camera, and the signal intensities were quantified and analyzed using Compass Software (ProteinSimple). During analysis, the signals for PKC δ were normalized to those for β -actin and the signals for PKC β II were normalized to those for β -actin or α -tubulin (loading controls) in the same samples. The normalized values for the drug treated samples were expressed relative to that of the DMSO treated control.

Western blot analysis

Total cell lysates, in ice cold phosphate buffered saline solution (PBS) containing 1% Triton X-100 and supplemented with protease and phosphatase inhibitors (Roche, Indianapolis, IN), were prepared by vortexing and sonication (3 times 6 sec). The preparation of nuclear extracts was performed as described earlier.(43) Samples containing 20 µg protein (protein concentration measured using the BIO-RAD DC^{Tm} protein assay) were added to SDS and beta-mercaptoethanol containing sample buffer (Quality Biological Inc), separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (Whatman GMBH). 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 h in the secondary antibody, and washed (3 times 5 min in PBS with 0.05% Tween-20). The signal was developed by ECL (Amersham) and detected on high performance chemiluminescence film (Amersham). The scanned films were edited using Adobe Photoshop CS3 (Adobe Systems).

Membrane fractionation of the treated LNCaP cells was performed by ultracentrifugation as described earlier.(51).

Translocation of different GFP-PKC isoforms

Translocation was detected as described earlier.(20) The human GFP-PKC δ was cloned similarly to mouse GFP-PKC δ as described earlier.(19) The mouse GFP-PKC α and mouse GFP-PKC ϵ constructs were described earlier (55), and the human GFP-PKC α , cloned similarly to mouse GFP-PKC α , was a gift from Dr. Chaya Brodie (Henry Ford Hospital, Michigan). The human PKC ϵ gene was PCR amplified (Origene) and cloned into the XhoI and AgeI sites of pYFP-YFP vector, creating the C- and N-terminally YFP-tagged PKC ϵ . The pYFP-YFP vector was created by insertion of EYFP gene amplified from pEYFP-C1 (Clontech) into the pEYFP-N1 plasmid (Clontech) using NheI and XhoI sites. All new constructs were verified by sequencing (DNA minicore, Center for Cancer Research, NCI, National Institutes of Health, Bethesda).

Real time RT-PCR analysis

RNA was isolated from cultured LNCaP cells with TRIzol reagent following the manufacturer's protocol (Invitrogen). For cDNA synthesis, 1.5 µg of total RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). Real time PCR was performed on MyiQ or iQ5 instruments (BIO-RAD) in a volume of 20 µl using iQ SYBR Green Supermix from BIORAD on 150 times diluted cDNA. The primers used were predesigned Quantitect primers from Qiagen (Valencia, CA) for the genes GAPDH, ALOXE3, BIRC3, CCL2, DUSP10, GATA2, CXCL8, MMP3, PPP1R3D, RAP2B, SERPINB2, SLC25A18, TIMP1, TNF-alpha, and TRAF1. Relative gene expression levels were calculated using the $2^{-(\Delta C_t)}$ formula, where ΔC_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.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

U937 cells (plated at 250,000 cell/ml) were stained with 40 nM MitoTracker Red CMXRos for 30 min at 37 °C after 24 h treatment with the different drugs and the fluorescent signal was detected by flow cytometry on a FACSCalibur (BD Biosciences) following the instructions of the manufacturer (Invitrogen). The data were analyzed by FlowJo software (TreeStar, Inc) and the results were expressed as percentage of cells showing reduced $\Delta\Psi_m$ as determined by reduced staining.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Effect of lipophilic substitution of some PKC ligands on their biological activity

Figure 2. Biological responses of U937 leukemia cells to PMA, bryostatin 1 or bryostatin 7 Growth (A), attachment (B) and TNF-alpha secretion (C) were measured after treatment with the indicated concentrations of PMA, bryostatin 1, bryostatin 7, or combinations thereof for 60 hours (A, B) or 24 hours (C). For measurements of growth and attachment, the numbers of attached and floating cells were counted using a Coulter Counter (A, B); secreted TNF-alpha levels were measured by ELISA (C). Ability to synergize with araC (1 μ M) for reduction in mitochondrial membrane potential was measured by flow cytometry after 24 h treatment with bryostatin 1 or bryostatin 7 (100 nM and 1000 nM, respectively) (D) Values represent the mean $\pm$ SEM of five (A, B), three (C) or four (D) independent experiments. Growth (A) was expressed relative to the vehicle control. Secreted TNF-alpha (C) was expressed relative to the level induced by 1000 nM PMA.

Figure 3. Down regulation of PKC β II (A) and PKC δ (B) in U937 cells

Cells were treated with the indicated concentrations of PMA, bryostatin 1 or bryostatin 7 for 24 hours. Levels of PKC β II and PKC δ were quantitated in total cell lysates by Simple WesternTM (Simon) using anti-PKC β II and anti-PKC δ antibodies. Loading was normalized to β -actin or α -tubulin, which provided loading controls, and normalized values were expressed relative to that of the DMSO treated cells. Values represent the mean $\pm$ SEM of three independent experiments. The lower panels show representative images of immunoblots performed on total cell lysates using the same antibodies and visualized by chemiluminescence.

Figure 4. Biological response of LNCaP cells

A) LNCaP cells were treated with the indicated nM concentrations of PMA, bryostatin 7 (bryo 7) or bryostatin 1 (bryo 1) for 36 h. Cell proliferation, measured using an Incucyte, was expressed relative to the DMSO control, and the percent inhibition by the various treatments was plotted. B) Inhibition of cell proliferation by PMA (30 nM) was determined in the absence or presence of the indicated concentrations of bryostatin 7 or bryostatin 1. C) The levels of TNFα secreted into the medium were determined 24 h after treatment with the indicated concentrations of PMA, bryostatin 7, or bryostatin 1. Values are expressed relative to that in response to 10 nM PMA. D) Secretion of TNFα in response to 30 nM PMA was determined in the absence or presence of the indicated concentrations of bryostatin 7 or bryostatin 1. All values represent the mean ± SEM of triplicate independent experiments.

Figure 5. Translocation of GFP-PKC δ in LNCaP cells after treatment with PMA, bryostatin 1 or bryostatin 7

Cells transiently transfected with mouse (**A**) or human (**B**) GFP-PKC δ were treated with 1000 nM PMA, 1000 nM bryostatin 1 or 3000 nM bryostatin 7. The translocation pattern

was examined by confocal microscopy as a function of time. Each panel represents images typical of the experiments performed. The panels for PMA, bryostatin 1 and bryostatin 7 represent $n = 5$, 7 , and 6 experiments, respectively, for mouse PKC δ and $n = 7$, 7 , and 11 experiments, respectively, for human PKC δ .

Figure 6. Translocation of human YFP-PKC ϵ in LNCaP cells after treatment with PMA, bryostatin 1, or bryostatin 7

Cells transiently transfected with human YFP-PKC ϵ were treated with 1000 nM PMA, bryostatin 1 or bryostatin 7. The translocation pattern was examined by confocal microscopy as a function of time. Each panel represents images typical of the experiments performed. The panels for PMA, bryostatin 1 and bryostatin 7 represent $n = 5$, 7 , and 4 experiments, respectively.

Figure 7. Translocation of human GFP-PKC α in CHO cells after treatment with PMA, bryostatin 1, or bryostatin 7

Cells transiently transfected with human GFP-PKC α were treated with 1000 nM PMA, bryostatin 1 or bryostatin 7. The translocation pattern was examined by confocal microscopy as a function of time. Each panel represents images typical of the experiments performed. The panels for PMA, bryostatin 1 and bryostatin 7 represent $n = 4$, 6, and 4 experiments, respectively.

Figure 8. Effect of ligands on subcellular distribution of PKC and PKD1 in LNCaP cells and on PKC signaling

A) LNCaP cells were treated for 60 min with ligands at the indicated concentrations, the cells were lysed, subjected to subcellular fractionation, and the fractionated lysates subjected to Western blotting with the indicated specific antibodies. TBP and lamin B1 were included as nuclear markers; both were affected by the various ligands. Results are representative of 4 independent experiments. B) Aliquots of the above whole cell lysates were subjected to Western blotting with the indicated specific antibodies. Results are representative of 4 independent experiments.

Figure 9. Effect of ligands on PKC distribution in LNCaP cells

LNCaP cells were treated with PMA, bryostatin and bryostatin 7 at the indicated concentrations for 30 min followed by membrane fractionation. The total cell lysates, the cytosolic and the membrane fractions were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. Results are representative of 2 independent experiments.

Figure 10. Gene induction in LNCaP cells after treatment with PMA, bryostatin 1 or bryostatin 7

Real time qPCR analysis was performed for 14 genes (for the list of genes see Materials and Methods) on RNA prepared from cells treated for 6 hours with 1000 nM PMA, 1000 nM bryostatin 1, or 1000 nM bryostatin 7. Symbols represent average values of four independent measurements for each gene and the lines indicate the geometric mean values. Values are expressed relative to that of the DMSO control.

Scheme 1.
Total syntheses of bryostatins 7 and 1

Table 1

Comparison of binding affinities to PKC isoforms of phorbol ester, bryostatin 1, and bryostatin 7. Values represent the mean $\pm$ SEM of triplicate independent experiments.

	Mouse PKC α	Human PKC α	Human PKC β II	Human PKC δ	Human PKC ϵ
PDBu K_d , nM	0.3 $\pm$ 0.05	0.28 $\pm$ 0.02	0.20 $\pm$ 0.00033	0.33 $\pm$ 0.083	0.22 $\pm$ 0.053
Bryostatin 1 K_d , nM	0.48 $\pm$ 0.033	0.73 $\pm$ 0.053	0.42 $\pm$ 0.013	0.26 $\pm$ 0.023	0.24 $\pm$ 0.013
Bryostatin 7, K_d , nM	0.26 $\pm$ 0.063	0.44 $\pm$ 0.013	0.32 $\pm$ 0.013	0.21 $\pm$ 0.023	0.16 $\pm$ 0.013