ACS chemical biology

Articles

pubs.acs.org/acschemicalbiology

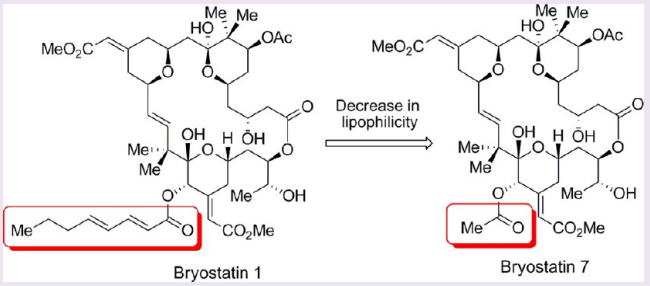
Biological Profile of the Less Lipophilic and Synthetically More Accessible Bryostatin 7 Closely Resembles That of Bryostatin 1

Noemi Kedei,[†] Nancy E. Lewin,[†] Tamás Géczy,[†] Julia Selezneva,[†] Derek C. Braun,[†] Jinqiu Chen,[‡] Michelle A. Herrmann,[‡] Madeleine R. Heldman,[‡] Langston Lim,[§] Poonam Mannan,[§] Susan H. Garfield,[§] Yam B. Poudel,[∥] Thomas J. Cummins,[∥] Arnab Rudra,[∥] Peter M. Blumberg,[†] and Gary E. Keck*[,∥]

[†]Laboratory of Cancer Biology and Genetics, [‡]Collaborative Protein Technology Resource, Laboratory of Cell Biology, [§]Laboratory of Experimental Carcinogenesis, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892, United States
[∥]Department of Chemistry, University of Utah, 315 South 1400 East, RM 2020, Salt Lake City, Utah 84112, United States

Supporting Information

ABSTRACT: The bryostatins are a group of 20 macrolides isolated by Pettit and co-workers 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 protein kinase C (PKC) 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.



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,[2−4] 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.[9−11]

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 20 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 PKCδ 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 PKCδ 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.[19−21]

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

Received: August 22, 2012
Accepted: January 18, 2013
Published: February 1, 2013

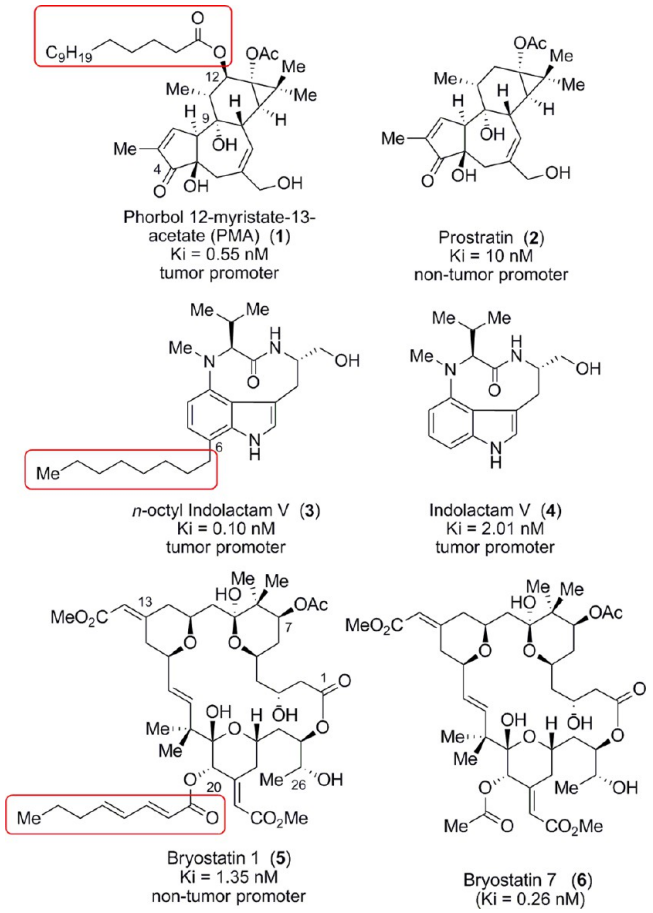


Figure 1. Effect of lipophilic substitution of some PKC ligands on their biological activity.

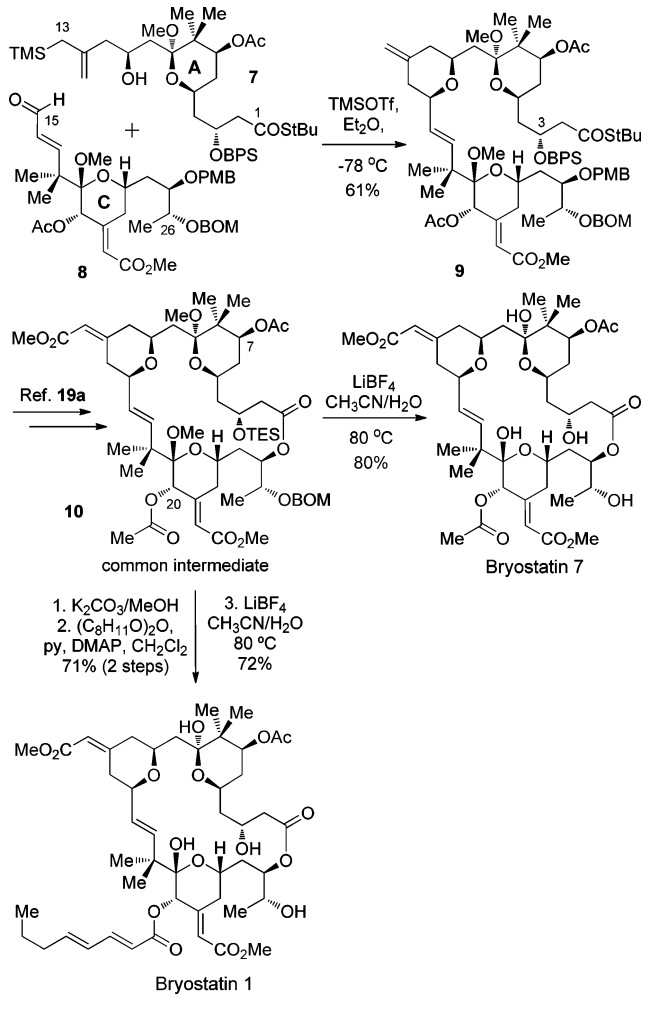
the most thoroughly studied member and has been subjected to numerous phase I and II clinical trials for cancer chemotherapy.[23] It has been shown to reverse multidrug resistance in cancer cells,[24] synergize with other oncolytic agents,[25−27] and stimulate the immune system.[28,29] Additionally, bryostatin 1 has exhibited promising neurological activity by improving learning and memory in animal models[30−32] 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 toward PKC (Figure 1).[35] Bryostatin 7 was isolated by Pettit and co-workers 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 co-workers, 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 resembles 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 $LiBF_4$ in

Scheme 1. Total Syntheses of Bryostatins 7 and 1



dx.doi.org/10.1021/cb300671s | ACS Chem. Biol. 2013, 8, 767−777

Table 1. Comparison of Binding Affinities (K_d, nM) to PKC Isoforms of Phorbol Ester, Bryostatin 1, and Bryostatin 7[a]

	mouse PKC α	human PKC α	human PKCβII	human PKC δ	human PKC ε
PDBu	0.3 ± 0.05	0.28 ± 0.02	0.20 ± 0.0003	0.33 ± 0.08	0.22 ± 0.05
bryostatin 1	0.48 ± 0.03	0.73 ± 0.05	0.42 ± 0.01	0.26 ± 0.02	0.24 ± 0.01
bryostatin 7	0.26 ± 0.06	0.44 ± 0.01	0.32 ± 0.01	0.21 ± 0.02	0.16 ± 0.01

[a]Values represent the mean ± SEM of triplicate independent experiments.

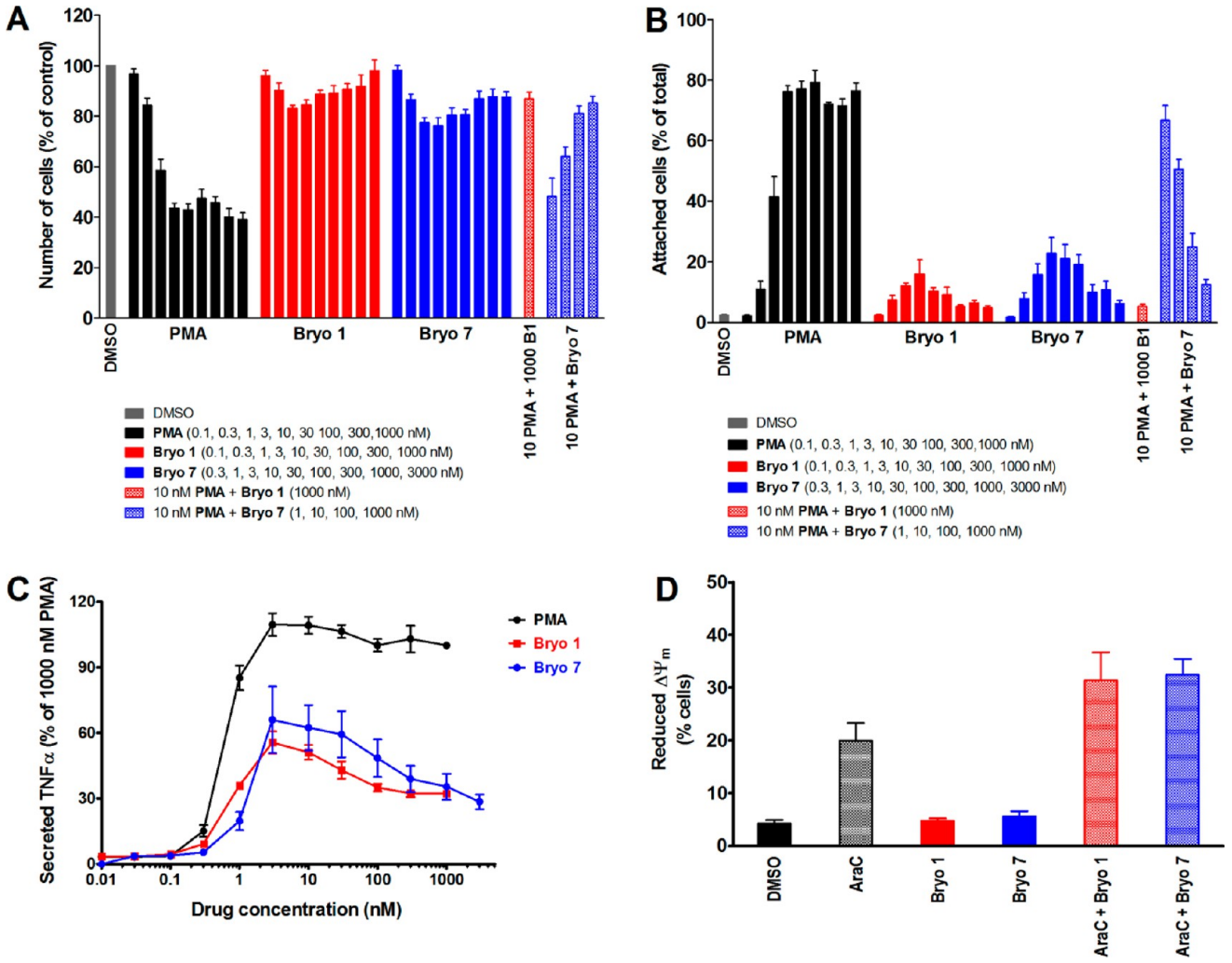


Figure 2. Biological responses of U937 leukemia cells to PMA, bryostatin 1, or bryostatin 7. Growth (A), attachment (B), and TNFα secretion (C) were measured after treatment with the indicated concentrations of PMA, bryostatin 1, bryostatin 7, or combinations thereof for 60 h (A, B) or 24 h (C). For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

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 K_2CO_3 in methanol for 45 min. 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 toward purified PKCα. Bryostatin 7 bound to mouse PKCα with a K_i of 0.26 ± 0.06 nM compared to a K_i of 0.48 ± 0.03 nM for bryostatin 1, assayed under the same conditions (Table 1). This result is of interest since the less lipophilic

dx.doi.org/10.1021/cb300671s | ACS Chem. Biol. 2013, 8, 767–777

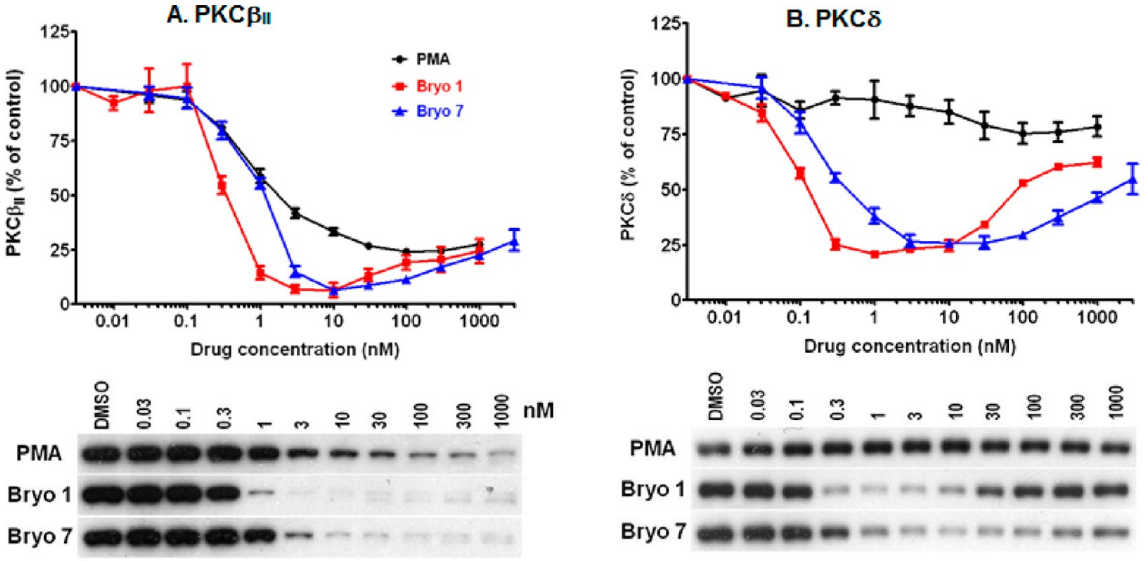


Figure 3. Down regulation of PKCβII (A) and PKCδ (B) in U937 cells. Cells were treated with the indicated concentrations of PMA, bryostatin 1, or bryostatin 7 for 24 h. Levels of PKCβII and PKCδ were quantitated in total cell lysates by Simple Western (Simon) using anti-PKCβII and anti-PKCδ antibodies. 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 ± 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.

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, βII, δ, and ε (Table 1). PKC βII, 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 analogues 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 (TNFα) in response to PMA, bryostatin 1, and bryostatin 7. TNFα 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 TNFα 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 PKCβII, PKCδ, and PKCε. Bryostatin 1 and 7 induced similar levels of down regulation of PKCβII, 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 ± 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 PKCβII signal at

dx.doi.org/10.1021/cb300671s | ACS Chem. Biol. 2013, 8, 767–777

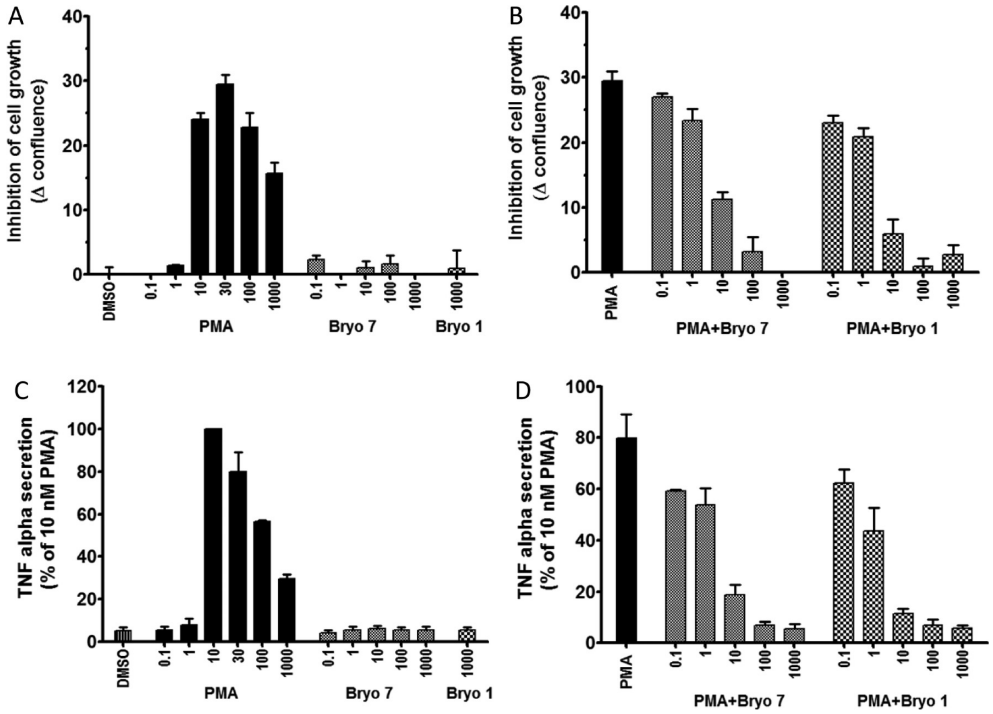


Figure 4. Biological response of LNCaP cells. (A) LNCaP cells were treated with the indicated nanomolar concentrations of PMA, bryostatin 7 (bryo 7), or bryostatin 1 (bryo 1) for 36 h. Cell proliferation, measured using an Incucyte, was expressed relative to the DMSO control, and the percent inhibition 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.

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 PKCβII.[48] For PKCδ, 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 ± 0.03 nM versus 0.083 ± 0.008 nM), indicating that bryostatin 7 and bryostatin 1 showed similar selectivity between PKCβII and PKCδ. PKCε 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 that 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 TNFα by PMA is thought to be a major mediator of this growth inhibition;[49] correspondingly, induction of TNFα by bryostatin 1 is much reduced.[43,49] 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 TNFα (Figure 4C). Like bryostatin 1, bryostatin 7 inhibits the secretion of TNFα induced by PMA (Figure 4D).

Translocation of GFP-PKCδ in LNCaP Cells. We have previously described that bryostatin 1 differed from PMA in the pattern of membrane translocation of PKCδ that it induced.[19] Using mouse PKCδ 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-PKCδ 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 PKCδ 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-PKCδ than did bryostatin 1. To test this prediction, we transiently transfected LNCaP cells with mouse GFP-PKCδ 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-PKCδ only to the nuclear membrane and internal membranes, whereas PMA caused initial translocation to the plasma membrane (Figure 5A).

dx.doi.org/10.1021/cb300671s | ACS Chem. Biol. 2013, 8, 767−777

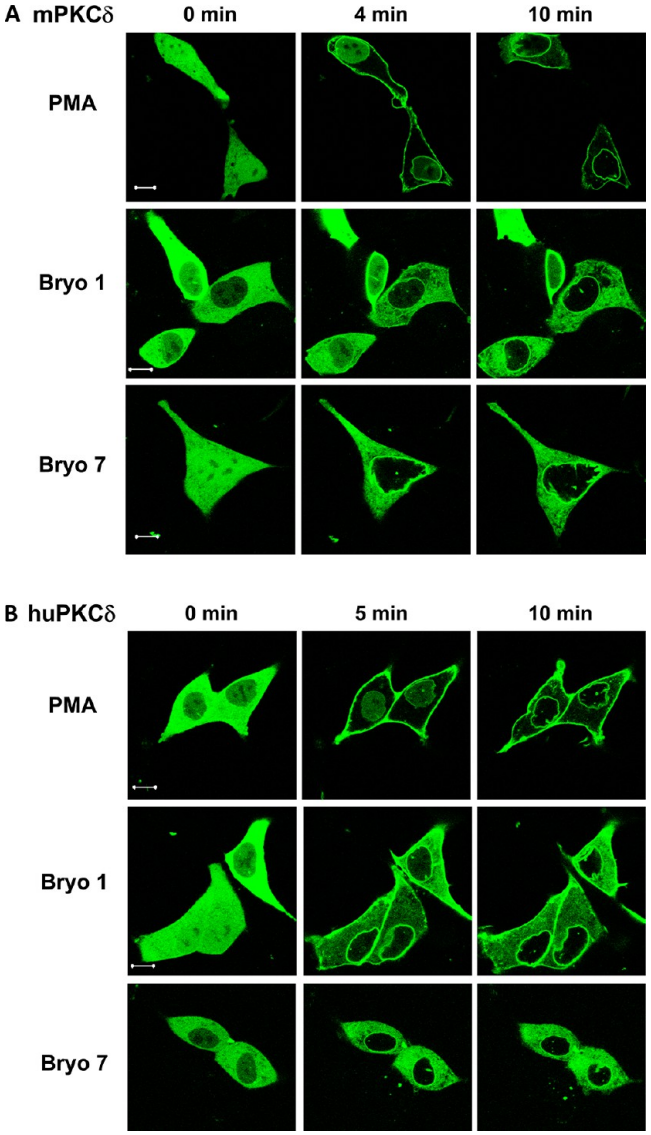


Figure 5. Translocation of GFP-PKCδ in LNCaP cells after treatment with PMA, bryostatin 1, or bryostatin 7. Cells transiently transfected with mouse (A) or human (B) GFP-PKCδ were treated with 1000 nM PMA, 1000 nM bryostatin 1, or 3000 nM bryostatin 7. The translocation pattern was examined by confocal 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 PKCδ and n = 7, 7, and 11 experiments, respectively, for human PKCδ.

Using transiently transfected human PKCδ rather than mouse PKCδ, 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-PKCδ 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-PKCε in LNCaP Cells. Because of the difference in the pattern of translocation of PKCδ in response to bryostatin 1 and bryostatin 7, we further compared the response of human YFP-PKCε to treatment with PMA, bryostatin 1, and bryostatin 7. The difference was dramatic. PMA caused rapid clearing of the cytoplasm with translocation of PKCε to the plasma membrane. Translocation was a little faster in the case of the mouse PKCε (data not shown) and a little slower in the case of the human PKCε (Figure 6).

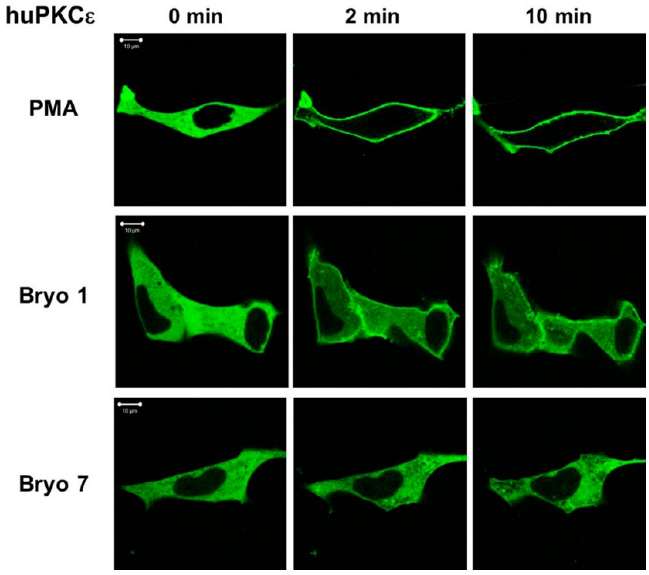


Figure 6. Translocation of human YFP-PKCε in LNCaP cells after treatment with PMA, bryostatin 1, or bryostatin 7. Cells transiently transfected with human YFP-PKCε were treated with 1000 nM PMA, bryostatin 1, or bryostatin 7. The translocation pattern was examined by confocal microscopy as a function 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.

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 PKCε in cancer and dementia, this differential activity by bryostatin 7 is particularly exciting.

Translocation of GFP-PKCα in Chinese Hamster Ovary (CHO) Cells. Unlike the novel PKC isoforms PCKδ and PKCε, PKCα 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-PKCα, 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-PKCα 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-PKCα (Figure 7). In contrast, bryostatin 1 induced a

dx.doi.org/10.1021/cb300671s | ACS Chem. Biol. 2013, 8, 767–777

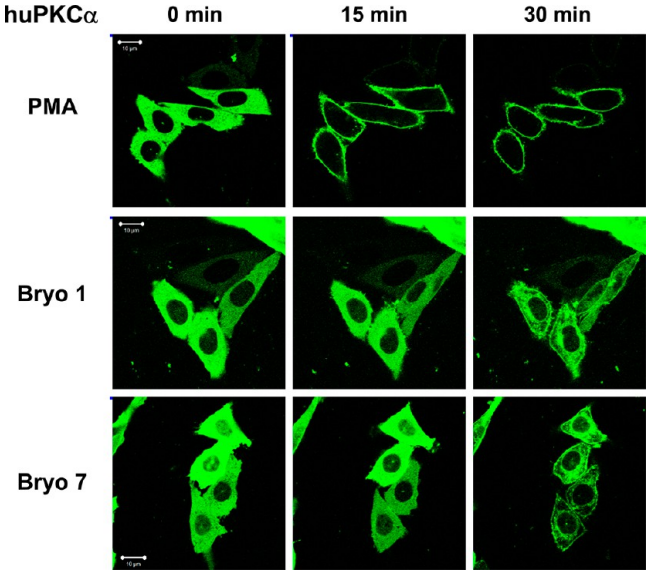


Figure 7. Translocation of human GFP-PKCα in CHO cells after treatment with PMA, bryostatin 1, or bryostatin 7. Cells transiently transfected with human GFP-PKCα were treated with 1000 nM PMA, bryostatin 1, or bryostatin 7. The translocation pattern was examined by confocal microscopy as a function of 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.

more complicated pattern, with some translocation of the human PKCα 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 PKCα 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-PKCα were similar to those for the human GFP-PKCα (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. PKCα and PKCε showed a similar shift to the nuclear enriched fraction in response to all three ligands. An increase in PKCδ in the nuclear enriched fraction was observed only for PMA. This difference between PMA and bryostatin 1 in accumulation of PKCδ in the nuclear enriched fraction had been reported by us previously;[43] bryostatin 7 resembled bryostatin 1 for this response. pPKD1 largely resembled PKCδ 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 PKCα, PKCδ, and PKCε 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

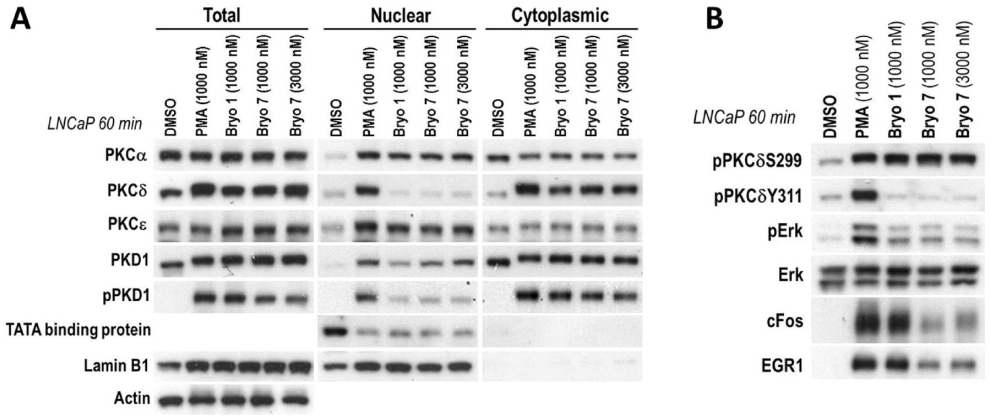


Figure 8. Effect of ligands on subcellular distribution of PKC and PKD1 in LNCaP cells and on PKC signaling. (A) LNCaP cells were treated for 60 min with ligands at the indicated concentrations, the cells were lysed and subjected to subcellular fractionation, and the fractionated lysates were subjected to Western blotting with the indicated specific antibodies. TATA binding protein 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.

dx.doi.org/10.1021/cb300671s | ACS Chem. Biol. 2013, 8, 767–777

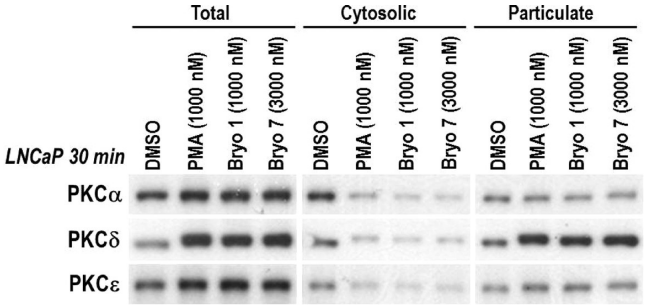


Figure 9. Effect of ligands on PKC distribution in LNCaP cells. LNCaP cells were treated with PMA, bryostatin 1 and bryostatin 7 at the indicated concentrations for 30 min followed by membrane fractionation. The total cell lysates and the cytosolic and the membrane fractions were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. Results are representative of 2 independent experiments.

bryostatin 7 for responses downstream of PKC activation (Figure 8B). Bryostatin 7 and bryostatin 1 were closely similar for induction of PKCδ phosphorylation at S299, a measure of activated PKCδ, and for their inability to induce efficient PKCδ 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

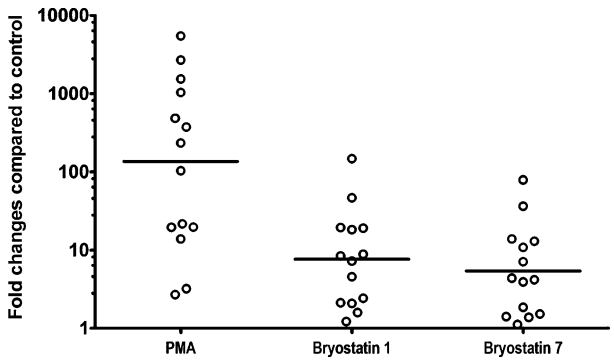


Figure 10. Gene induction in LNCaP cells after treatment with PMA, bryostatin 1, or bryostatin 7. Real time qPCR analysis was performed for 14 genes (for the list of genes see Methods) on RNA prepared from cells treated for 6 h with 1000 nM PMA, 1000 nM bryostatin 1, or 1000 nM bryostatin 7. Symbols 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.

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 h than at 3 h 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 PKCδ translocation that it induced, where there was even less plasma membrane translocation than seen with bryostatin 1. The selectivity between PKCβII and PKCδ 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 PKCε 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 lipohilicity of bryostatin 7 may be advantageous. Moreover, these results very clearly show that the long C20 side chain 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 RPM1-1640 medium were obtained from ATCC. Precast 10% SDS gels, TNFα ELISA kit, SuperScript II Reverse Transcriptase, PBS, MitoTracker Red CMXRos, and the human PKC isoforms were from Invitrogen. The primary antibodies against PKCβII (sc-210), PKCδ (sc-937), PKCε (sc-214), and cFos (sc-7202) were from Santa Cruz Biotechnology, and the ones against PKCα (C-terminal), pPKCδS299, pPKCδY311, 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 nonfat 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.

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 and 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.

Growth of LNCaP Cells. Growth was determined using an Incucyte instrument as described earlier.[43]

Measurement of TNFα. 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 and then centrifuged (4000 rpm for 5 min in an Eppendorf microcentrifuge) to remove any cells, and TNFα 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 min. 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 min 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-PKCδ or anti-PKCβII 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 PKCδ were normalized to those for β-actin and the signals for PKCβII 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 s). The preparation of nuclear extracts was performed as described earlier.[43] Samples containing 20 μg protein (protein concentration measured using the BIO-RAD *DC* protein assay) were added to SDS and β-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. 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-PKCδ was cloned similarly to mouse GFP-PKCδ as described earlier.[19] The mouse GFP-PKCα and mouse GFP-PKCε constructs were described earlier,[55] and the human GFP-PKCα, cloned similarly to mouse GFP-PKCα, was a gift from Dr. Chaya Brodie (Henry Ford Hospital, Michigan). The human PKCε 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 PKCε. 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, TNFα, and TRAF1. Relative gene expression levels were calculated using the $2^{-(\Delta Ct)}$ formula, where ΔCt 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.

■ ASSOCIATED CONTENT

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.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: keck@chem.utah.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by the National Institutes of Health through Grant GM28961 to G.K. and in part by the Intramural Research Program of the National Institutes of Health, Center for Cancer Research, National Cancer Institute (Z1A BC 005270). We thank C. Brodie for providing the human GFP-PKCα plasmid.

■ REFERENCES

(1) Murray, N. R., Thompson, L. J., and Fields, A. P. (1997) The role of protein kinase C in cellular proliferation and cell cycle control, in *Molecular Biology Intelligence Unit* (Parker, P. J. and Dekker, L. Eds.), pp 97−120, R. G. Landes Co., Austin, TX.

(2) Griner, E. M., and Kazanietz, M. G. (2007) Protein kinase C and other diacylglycerol effectors in cancer. *Nat. Rev. Cancer. 7*, 281−294.

(3) Mackay, H. J., and Twelves, C. J. (2007) Targeting the protein kinase C family: Are we there yet? *Nat. Rev. Cancer 7*, 554−562.

(4) Teicher, B. A. (2006) Protein kinase C as a therapeutic target. *Clin. Cancer Res. 12*, 5336−5345.

(5) Etcheberrigaray, R., Tan, M., Dewachter, I., Kuiperi, C., Van der Auwera, I., Wera, S., Qiao, L., Baank, B., Nelson, T. J., Kozikowski, A. P., Van Leuven, F., and Alkon, D. L. (2004) Therapeutic effects of PKC activators in Alzheimer's disease transgenic mice. *Proc. Natl. Acad. Sci. U.S.A. 101*, 11141−11146.

(6) Ishii, H., Jirousek, M. R., Koya, D., Takagi, C., Xia, P., Clermont, A., Bursell, S. E, Kern, T. S, Ballas, L. M, Heath, W. F., Stramm, L. E., Feener, E. P., and King, G. L. (1996) Amelioration of vascular dysfunctions in diabetic rats by an oral PKCb inhibitor. *Science 272*, 728−731.

(7) Roebuck, K. A., Gu, D. S., and Kagnoff, M. F. (1996) Activating protein-1 cooperates with phorbol ester activation signals to increase HIV-1 expression. *AIDS 10*, 819−826.

(8) Murphy, S., and Frishman, W. H. (2005) Protein kinase C in cardiac disease and as a potential therapeutic target. *Cardiol. Rev. 13*, 3−12.

(9) Andrejauskas-Buchdunger, E., and Regenass, U. (1992) Differential inhibition of the epidermal growth factor-, platelet-derived growth factor-, and protein kinase C-mediated signal transduction pathways by the staurosporine derivative CGP 41251. *Cancer Res. 52*, 5353−5358.

(10) Teicher, B. A., Alvarez, E., Menon, K., Esterman, M. A., Considine, E., Shih, C., and Faul, M. M. (2002) Antiangiogenic effects of a protein kinase Cb-selective small molecule. *Cancer Chemother. Pharmacol. 49*, 69−77.

(11) Marquez, V. E., and Blumberg, P. M. (2003) Synthetic diacylglycerols (DAG) and DAG-lactones as activators of protein kinase C (PK-C). *Acc. Chem. Res. 36*, 434−443.

(12) Zhang, G., Kazanietz, M. G., Blumberg, P. M., and Hurley, J. H. (1995) Crystal structure of the cys2 activator-binding domain of protein kinase C delta in complex with phorbol ester. *Cell 81*, 917−924.
(13) Heikkilä, J., and Akerman, K. E. (1989) (−)-Indolactam V activates protein kinase C and induces changes in muscarinic receptor functions in SH-SY5Y human neuroblastoma cells. *Biochem. Biophys. Res. Commun. 15*, 1207−1213.
(14) Hecker, E. (1968) Cocarcinogenic principles from the seed oil of Croton tiglium and from other Euphorbiaceae. *Cancer Res. 28*, 2338−2349.
(15) Bögi, K., Lorenzo, P. S., Szállási, Z., Acs, P., Wagner, G. S., and Blumberg, P. M. (1998) Differential selectivity of ligands for the C1a and C1b phorbol ester binding domains of protein kinase Cdelta: possible correlation with tumor-promoting activity. *Cancer Res. 58*, 1423−1428.
(16) Szallasi, Z., Krsmanovic, L., and Blumberg, P. M. (1993) Nonpromoting 12-deoxyphorbol 13-esters inhibit phorbol 12-myristate 13-acetate induced tumor promotion in CD-1 mouse skin. *Cancer Res. 53*, 2507−2512.
(17) Zayed, S., Sorg, B., and Hecker, E. (1984) Structure activity relations of polyfunctional diterpenes of the tigliane type, VI. Irritant and tumor promoting activities of semisynthetic mono and diesters of 12-deoxyphorbol. *Planta Med. 50*, 65−69.
(18) Nakagawa, Y., Irie, K., Nakamura, Y., Ohigashi, H., and Hayashi, H. (1998) Synthesis and biological activities of (−)-6-n-octyl-indolactam-V, a new potent analogue of the tumor promoter (−)-indolactam-V. *Biosci. Biotechnol. Biochem. 62*, 1568−1573.
(19) Wang, Q. J., Bhattacharyya, D., Garfield, S., Nacro, K., Marquez, V. E., and Blumberg, P. M. (1999) Differential localization of protein kinase C delta by phorbol esters and related compounds using a fusion protein with green fluorescent protein. *J. Biol. Chem. 274*, 37233−37239.
(20) Kedei, N., Lubart, E., Lewin, N. E., Telek, A., Lim, L., Mannan, P., Garfield, S. H., Kraft, M. B., Keck, G. E., Kolusheva, S., Jelinek, R., and Blumberg, P. M. (2011) Some phorbol esters might partially resemble bryostatin 1 in their actions on LNCaP prostate cancer cells and U937 leukemia cells. *ChemBioChem 12*, 1242−1251.
(21) Wang, Q. J., Fang, T. W., Fenick, D., Garfield, S., Bienfait, B., Marquez, V. E., and Blumberg, P. M. (2000) The lipophilicity of phorbol esters as a critical factor in determining the pattern of translocation of protein kinase C delta fused to green fluorescent protein. *J. Biol. Chem. 275*, 12136−12146.
(22) Kazanietz, M. G., Lewin, N. E., Gao, F., Pettit, G. R., and Blumberg, P. M. (1994) Binding of [26-^{3}H]bryostatin 1 and analogs to calcium-dependent and calcium-independent protein kinase C isozymes. *Mol. Pharmacol. 46*, 374−379.
(23) For further information, see: http://www.clinicaltrials.gov/
(24) Spitaler, M., Utz, I., Hilbe, W., Hofmann, J., and Grunicke, H. H. (1998) PKC-independent modulation of multidrug resistance in cells with mutant (V185) but not wild-type (G185) p-glycoprotein by bryostatin 1. *Biochem. Pharmacol. 56*, 861−869.
(25) Koutcher, J. A., Motwani, M., Zakian, K. L., Li, X.−K., Matei, C., Dyke, J. P., Ballon, D., Yoo, H.−H., and Schwartz, G. K. (2000) The *in vivo* effect of bryostatin-1 on paclitaxel-induced tumor growth, mitotic entry, and blood flow. *Clin. Cancer Res. 6*, 1498−1507.
(26) Basu, A., and Lazo, J. S. (1992) Sensitization of human cervical carcinoma cells to *cis*-diaminedichloroplatinum(II) by bryostatin 1. *Cancer Res. 52*, 3119−3124.
(27) Mohammad, R. M., Wall, N. R., Dutcher, Julie, A., and Al-Katib, A. M. (2000) The addition of bryostatin 1 to cyclophosphamide, doxorubicin, vincristine, and prednisone (chop) chemotherapy improves response in a chop-resistant human diffuse large cell lymphoma xenograft model. *Clin. Cancer Res. 6*, 4950−4956.
(28) Berkow, R. L., and Kraft, A. S. (1985) Bryostatin, a non-phorbol macrocyclic lactone, activates intact human polymorphonuclear leukocytes and binds to the phorbol ester receptor. *Biochem. Biophys. Res. Commun. 131*, 1109−1116.
(29) Bosco, M. C., Rottschafer, S., Taylor, L. S., Ortaldo, J. R., Longo, D. L., and Espinoza-Delgado, I. (1997) The antineoplastic agent bryostatin-1 induces proinflammatory cytokine production in human monocytes: synergy with interleukin-2 and modulation of interleukin-2rγ chain expression. *Blood 89*, 3402−3411.
(30) Sun, M.-K., and Alkon, D. L. (2005) Dual effects of bryostatin-1 on spatial memory and depression. *Eur. J. Pharmacol. 512*, 43−51.
(31) Kuzirian, A. M., Epstein, H. T., Gagliardi, C. J., Nelson, T. J., Sakakibara, M., Taylor, C., Scioletti, A. B., and Alkon, D. L. (2006) Bryostatin enhancement of memory in *Hermissenda*. *Biol. Bull. 210*, 201−214.
(32) Sun, M.-K., and Alkon, D. L. (2008) Synergistic effects of chronic bryostatin-1 and α-tocopherol on spatial learning and memory in rats. *Eur. J. Pharmacol. 584*, 328−337.
(33) For further information, see: http://www.brni.org/
(34) Mehla, R., Bivalkar-Mehla, S., Zhang, R., Handy, I., Albrecht, H., Giri, S., Nagarkatti, P., Nagarkatti, M., and Chauhan, A. (2012) Bryostatin modulates latent HIV-1 infection via PKC and AMPK signaling but inhibits acute infection in a receptor independent manner. *PLoS One 5*, e11160.
(35) Wender, P. A., Cribbs, C. M., Koehler, K. F., Sharkey, N. A., Herlad, C. L., Kamano, Y., Pettit, G. R., and Blumberg, P. M. (1988) Modeling of the bryostatins to the phorbol ester pharmacophore on protein kinase C. *Proc. Natl. Acad. Sci. U.S.A. 85*, 7197−7201.
(36) Pettit, G. R., Kamano, Y., Herald, C. L., and Tozawa, M. (1985) Isolation and structure of bryostatins 5−7. *Can. J. Chem. 63*, 1204−1208.
(37) Tallant, E. A., Smith, J. B., and Wallace, R. W. (1987) Bryostatins mimic the effects of phorbol esters in intact human platelets. *Biochim. Biophys. Acta 929*, 40−46.
(38) Carr, M. E., Jr., Carr, S. L., and Grant, S. (1995) A sensitive platelet activation-based functional assay for the antileukemic agent bryostatin 1. *Anticancer Drugs 6*, 384−391.
(39) Keck, G. E., Poudel, Y. B., Cummins, T. J., Rudra, A., and Covel, J. A. (2011) Total synthesis of bryostatin 1. *J. Am. Chem. Soc. 133*, 744−747.
(40) Keck, G. E., Covel, J. A., Schiff, T., and Yu, T. (2002) Pyran annulation: asymmetric synthesis of 2,6-disubstituted-4-methylene tetrahydropyrans. *Org. Lett. 4*, 1189−1192.
(41) Lipschutz, B. H., and Harvey, D. F. (1982) Hydrolysis of acetals and ketals using $LiBF_4$. *Synth. Commun. 14*, 267−277.
(42) Kedei, N., Telek, A., Michalowski, A. M., Kraft, M. B., Li, W., Poudel, Y .B., Rudra, A., Petersen, M. E., Keck, G. E., and Blumberg, P. M. (2012) 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. *Biochem. Pharmacol. 85*, 313−324.
(43) Kedei, N., Telek, A., Czap, A., Lubart, E. S., Czifra, G., Yang, D., Chen, J., Morrison, T., Goldsmith, P. K., Lim, L., Mannan, P., Garfield, S. H., Kraft, M. B, Li, W., Keck, G. E, and Blumberg, P. M. (2011) The synthetic bryostatin analog Merle 23 dissects distinct mechanisms of bryostatin activity in the LNCaP human prostate cancer cell line. *Biochem. Pharmacol. 81*, 1296−1308.
(44) Kazanietz, M. G., Areces, L. B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J. F., and Blumberg, P. M. (1993) Characterization of ligand and substrate specificity for the calcium-dependent and calcium-independent protein kinase C isozymes. *Mol. Pharmacol. 44*, 296−307.
(45) Vrana, J. A., Saunders, A. M., Chellappan, S. P., and Grant, S. (1998) Divergent effects of bryostatin 1 and phorbol myristate acetate on cell cycle arrest and maturation in human myelomonocytic leukemia cells (U937). *Differentiation 63*, 33−42.
(46) Meinhardt, G., Roth, J., and Totok, G. (2000) Protein kinase C activation modulates pro- and anti-apoptotic signaling pathways. *Eur. J Cell Biol. 79*, 824−833.
(47) Wang, S., Wang, Z., and Grant, S. (2003) Bryostatin 1 and UCN-01 potentiate 1-β-D-arabinofuranosylcytosine-induced apoptosis in human myeloid leukemia cells through disparate mechanisms. *Mol. Pharmacol. 63*, 232−242.

(48) Gould, C. M., and Newton, A. C. (2008) The life and death of protein kinase C. *Curr. Drug Targets 9*, 614−625.
(49) von Burstin, V. A., Xiao, L., and Kazanietz, M. G. (2010) Bryostatin 1 inhibits phorbol ester-induced apoptosis in prostate cancer cells by differentially modulating protein kinase C (PKC) delta translocation and preventing PKC delta-mediated release of tumor necrosis factor-alpha. *Mol. Pharmacol. 78*, 325−332.
(50) Leontieva, O. V., and Black, J. D. (2004) Identification of two distinct pathways of protein kinase C alpha down-regulation in intestinal epithelial cells. *J. Biol. Chem. 279*, 5788−5801.
(51) Braun, D. C., Garfield, S. H., and Blumberg, P. M. (2005) Analysis by fluorescence resonance energy transfer of the interaction between ligands and protein kinase Cdelta in the intact cell. *J. Biol. Chem. 280*, 8164−8171.
(52) Caino, M. C., von Burstin, V. A., Lopez-Haber, C., and Kazanietz, M. G. (2011) Differential regulation of gene expression by protein kinase C isozymes determined by genome-wide expression analysis. *J. Biol. Chem. 286*, 11254−11264.
(53) Keck, G. E., Kraft, M. B., Truong, A. P., Li, W., Sanchez, C. C., Kedei, N., Lewin, N., and Blumberg, P. M. (2008) Convergent assembly of highly potent analogues of bryostatin 1 via pyran annulation: bryostatin look-alikes that mimic phorbol ester function. *J. Am. Chem. Soc. 130*, 6660−6661.
(54) Nguyen, U., Squaglia, N., Boge, A., and Fung, P. A. (2011) The Simple Western: a gel-free, blot-free, hands-free Western blotting reinvention. *Nat. Methods 8*, v−vi.
(55) Kedei, N., Lundberg, D. J., Toth, A., Welburn, P., Garfield, S. H., and Blumberg, P. M. (2004) Characterization of the interaction of ingenol 3-angelate with protein kinase C. *Cancer Res. 64*, 3243−3255.