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Some phorbol esters may partially resemble bryostatin 1 in their actions on LNCaP prostate cancer cells and U937 leukemia cells

Noemi Kedei, M.D.^[a][staff scientist], Emanuel Lubart, B.S.^[a][CRTA fellow], Nancy E. Lewin, B.S.^[a][Senior Research Assistant], Andrea Telek, M.D., Ph.D.^[a][Postdoctoral Fellow], Langston Lim^[b][Laboratory Biologist], Poonam Mannan, M.S.^[b][Laboratory Biologist], Susan H. Garfield, M.S.^[b][Core Facility Head], Matthew B. Kraft^[c][Research Assistant], Gary E. Keck, Ph.D.^[c][Professor], Sofiya Kolusheva, Ph.D.^[d], Raz Jelinek, Ph.D.^[d][Professor], and Peter M. Blumberg, Ph.D.^{*,[a]}[Laboratory chief]

^[a] Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, U.S.A.

^[b] Laboratory of Experimental Carcinogenesis, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, U.S.A.

^[c] Department of Chemistry, University of Utah, Salt Lake City, UT 84112, U.S.A.

^[d] Department of Chemistry, Ben Gurion University, Beer Sheva 84105, Israel

Abstract

Phorbol 12-myristate 13-acetate (PMA) and bryostatin 1 are both potent protein kinase C (PKC) activators. In LNCaP human prostate cancer cells, PMA induces tumor necrosis factor alpha (TNF α) secretion and inhibits proliferation; bryostatin 1 does not and itself blocks the response to PMA. This difference has been attributed to bryostatin 1 not localizing PKC δ to the plasma membrane. Since phorbol ester lipophilicity influences PKC δ localization, we have examined in LNCaP cells a series of phorbol esters and related derivatives spanning some 8 logs in lipophilicity (LogP) to see if any behave like bryostatin 1. The compounds showed marked differences in their effects on proliferation and TNF α secretion. For example, maximal responses for TNF α secretion relative to PMA ranged from 97% for octyl-indolactam V to 24% for phorbol 12,13-dibenzoate. Dose response curves ranged from monophasic for indolactam V to markedly biphasic for sapintoxin D. The divergent patterns of response, however, correlated neither with lipophilicity, with plasma membrane translocation of PKC δ , or with the ability to interact with model membranes. In U937 human leukemia cells, a second system in which PMA and bryostatin 1 have divergent effects, viz. PMA but not bryostatin 1 inhibits proliferation and induces attachment, all the compounds acted like PMA for proliferation but several induced a reduced level or a biphasic dose response curve for attachment. We conclude that active phorbol esters are not all equivalent. Depending on the system, some may partially resemble bryostatin 1 in their behavior, encouraging the concept that bryostatin-like behavior may be obtained from other structural templates.

*To whom correspondence should be addressed at: National Cancer Institute, Building 37, Room 4048B, 37 Convent Drive MSC 4255 Bethesda, MD 20892-4255 Tel: 301-496-318 FAX: 301-496-8709 blumberg@dc37a.nci.nih.gov.

Noemi Kedei M.D. staff scientist, Emanuel Lubart B.S. CRTA fellow, Nancy E. Lewin B.S. Senior Research Assistant, Andrea Telek M.D., Ph.D. Postdoctoral Fellow, Langston Lim Laboratory Biologist, Poonam Mannan M.S. Laboratory Biologist, Susan H. Garfield, M.S. Core Facility Head, Matthew B. Kraft Research Assistant, Gary E. Keck Ph.D. Professor, Sofiya Kolusheva Ph.D., Raz Jelinek Ph.D. Professor, Peter M. Blumberg Ph.D. Laboratory chief

Keywords

lipophilicity; biological activity; protein kinase C; PMA; bryostatin 1

Introduction

Protein kinase C (PKC) plays a critical role in integrating and interpreting the signals of the multiple cellular receptors which respond to ligand binding by phospholipase C activation, resulting in breakdown of phosphoinositides and the generation of the lipophilic second messenger sn-1,2diacylglycerol (DAG).^[1] DAG binds to the C1 domains of PKC, both causing enzyme activation and driving its translocation to cellular membranes. This localization, in turn, influences enzyme specificity by controlling the substrates with which it is colocalized. As might be expected from the central role of PKC in cellular signal processing, PKC has emerged as a promising therapeutic target for cancer.^[2] Complementary drug strategies targeting PKC are reflected in inhibitors of PKC catalytic activity such as enzastaurin,^[3] inhibitors of PKC expression such as the antisense Aprinocarsen,^[4] and potent natural product mimetics of DAG such as ingenol 3-angelate (PEP005)^[5] and bryostatin 1.^[6]

Bryostatin 1 has attracted considerable attention as a consequence of its paradoxical activity. Whereas it binds to PKC with high affinity, leading to enzyme activation, in many biological systems it behaves as a functional antagonist of PMA. It fails to induce the responses observed for PMA and, when co-applied with PMA, it blocks the PMA responses, confirming that the bryostatin 1 is not simply inactive or unstable.^[7] On-going synthetic efforts with bryostatin derivatives seek to understand the structural features conferring its unique pattern of activity.^[8] Among the biological systems in which the different responses of bryostatin 1 and PMA have been characterized are the LNCaP human prostate cancer cell line^[9] and the U937 human leukemia cell line.^[10] In the LNCaP cells, whereas PMA induces apoptosis,^[11] in part driven by stimulation of secretion of TNF α ,^[9c, 12] bryostatin 1 does not.^[9] In the U937 cells, PMA inhibits proliferation and induces cell attachment, whereas bryostatin 1 has a much reduced effect.^[10] In both systems, bryostatin 1 antagonizes the effect of PMA, confirming that bryostatin 1 is not simply inactive in these systems but rather actively antagonizes the action of PMA. Mechanistic analysis by von Burstin and Kazanietz^[9c] suggested that the basis for the difference in behavior of PMA and bryostatin 1 in the LNCaP cells was that PMA induced plasma membrane translocation of PKC delta, whereas bryostatin 1 did not. In support of this conclusion, when they expressed in the LNCaP cells a modified PKC delta which had a myristoylation site incorporated at its C terminus to drive plasma membrane association, bryostatin 1 then behaved like PMA, inducing TNF α secretion and growth inhibition.

We have previously described in Chinese hamster ovary cells that PMA caused PKC delta to translocate initially to the plasma membrane, followed by partial redistribution to the nuclear membrane and other internal membranes; bryostatin 1, in contrast, failed to cause initial translocation to the plasma membrane but rather caused translocation to internal membranes directly.^[13] Examination of a series of phorbol esters and related derivatives showed that the lipophilicity of the compounds determined the pattern of translocation of PKC δ , with more lipophilic compounds behaving like PMA and less lipophilic compounds behaving like bryostatin 1.^[14] If, as suggested by von Burstin and Kazanietz,^[9c] the failure of bryostatin 1 to induce TNF α secretion and inhibit proliferation in the LNCaP cells resulted from the lack of plasma membrane translocation of PKC δ , a prediction is that less lipophilic phorbol esters should be bryostatin 1 mimetics.

In the present study, we evaluated a series of diverse phorbol esters and related PKC ligands in two well characterized systems in which bryostatin 1 has been shown to fail to induce prominent responses induced by PMA and to block those responses when administered together with PMA. These systems were chosen because of the differences distinguishing bryostatin 1 activity from that of typical PKC ligands that make bryostatin 1 of particular therapeutic interest. We address two related questions. Is the behavior of bryostatin 1 in these systems truly unique to bryostatin 1 or is it mimicked by some phorbol esters? Can the lipophilicity of the ligands (and their resultant differential localization of PKC delta) account for the patterns of biological response that they induce?

Results

We selected a series of commercially available phorbol esters, ingenols, indolactams and Sapintoxin D (Figure 1) which span close to 9 orders of magnitude in lipophilicity, with calculated LogP values ranging from 0.07 to 8.8 (Supplementary Table 1 and Figure 2). These values bracket the reported experimentally determined LogP value of bryostatin 1, which was 2.88.^[15] As a further criterion, we tried to select compounds with divergent structural features or unusual patterns of biological activity. Prostratin is anti-tumor promoting^[16] and has been suggested to be of therapeutic utility for abolition of HIV latency.^[17] Ingenol 3-angelate is in clinical trials for skin cancer,^[5, 18] differs from typical phorbol esters in the pattern of biological response both in WEHI-231 cells^[19] and in mouse skin,^[20] and, as in ingenol 3-ester, has its hydrophobic domain differently oriented relative to the diterpene moiety compared to the phorbol esters.^[21] Sapintoxin D has been used to define differential binding affinities for the C1 domains of PKC and has weak tumor promoting activity.^[22] Phorbol 12,13-dibenzoate is very weak as a tumor promoter.^[23] Indolactams are the simplest members of the teleocidin family with highly inflammatory and tumor promoter activity.^[24] All of the compounds are ligands for PKC, binding to the C1 domain competitively with phorbol 12,13-dibutyrate. Binding affinities for PKC varied somewhat, with reported K_i values ranging from 0.1 nM to 10 nM (65 nM for phorbol 12,13-diacetate).

In the LNCaP cells, PMA causes marked inhibition of cell growth at 72 hrs. In contrast, although bryostatin 1 is not without effect, the maximal level of inhibition was only 19.6 % of that for PMA (Figure 2, Table 1). These results are similar to those observed by us and others previously. Among the series of other phorbol esters and related derivatives, the first striking finding was that the pattern of response varied dramatically in the degree of their maximal response (Table 1). Several of the compounds, in particular the two indolactams, were even more effective than PMA at causing inhibition of growth. In contrast, phorbol 12,13-dibenzoate was only slightly more effective than bryostatin 1; maximal inhibition of growth was only 14 % greater. Several other derivatives, sapintoxin D and phorbol 12,13-dihexanoate, yielded an intermediate but still weak response, followed by prostratin, phorbol 12,13-dibutyrate, and phorbol 12,13-didecanoate. A second feature of the dose response curves was the extent to which they were biphasic. Phorbol 13-decanoate at a maximally effective dose (1000 nM) reached 92 % of the PMA responses, whereas a 10-fold higher dose gave no more inhibition than did bryostatin 1. In contrast, little biphasic response was seen for some of the other derivatives, such as indolactam V or phorbol 12,13-diacetate. We conclude that the failure to inhibit cell growth in the LNCaP cells is not limited to bryostatin 1 among PKC activators. Rather, PKC ligands show a continuum of extents of inhibition of LNCaP cell growth.

The induction of TNF α secretion is a major contributor, although not the sole factor, responsible for the inhibition of LNCaP cell growth in response to PMA. Roughly in parallel with their abilities to inhibit cell growth, the phorbol esters and related derivatives that we

examined varied in their abilities to induce TNF α secretion (Figure 3, Table 1). Thus, octylindolactam V induced TNF α to a level comparable to that induced by PMA, whereas phorbol 12,13-dibenzoate at its maximally effective dose induced only 24 % of that level. Sapintoxin D was somewhat more effective than expected from its weak inhibition of growth but the dose response curve was markedly biphasic, with a dose of 10,000 nM giving only 5 % of the maximal level of secretion induced by PMA. Once again, our data strongly argue that the phorbol esters and related derivatives are not all equivalent but vary markedly in the extents of TNF α secretion that they were able to induce. As with the findings for growth, however, all of the derivatives were at least modestly more effective for inducing TNF α secretion than was bryostatin 1.

When comparing the maximal extents of TNF α secretion induced by the various phorbol esters and related derivatives in the LNCaP cells with the calculated LogP values of the compounds we found that all of the compounds which induced a lower maximal response than did PMA possessed LogP values between two and five (Supplementary Table 1). On the other hand, some of the compounds in this LogP range induced levels of TNF α secretion similar to PMA, indicating that a LogP value within this range was not sufficient to endow the phorbol ester with reduced activity for inducing TNF α secretion.

We have previously characterized the pattern of PKC delta subcellular localization in Chinese hamster ovary cells in response to PMA, bryostatin 1 and a series of phorbol esters that varied in their lipophilicities.^[13-14] For direct comparison, we have determined how the ligands of the current study affect PKC delta localization in the LNCaP cells. Visualizing PKC delta localization using a GFP-PKC delta construct, we find that PKC delta responded more or less similarly in the LNCaP cells to what we had described earlier in the Chinese hamster ovary cells (Figure 4). PKC delta initially translocated to the plasma membrane in response to PMA, subsequently partially redistributing to the nuclear membrane and internal membranes. A similar pattern was observed for phorbol 12,13-decanoate, albeit with a slower rate of translocation. Bryostatin 1 caused modestly more plasma membrane translocation than we had observed in the Chinese hamster ovary cells. The less lipophilic ligands predominantly caused translocation to internal membranes, with little plasma membrane localization. Little difference was observed between compounds that caused more or less inhibition of growth / TNF α secretion.

The second biological system that we examined was that of the U937 human leukemia cells. In this system PMA inhibits cell growth, whereas bryostatin 1 again has a much reduced effect. All of the series of phorbol esters and related derivatives which we examined showed behavior basically similar to that of PMA (Figure 5, Table 1). PMA induced 54% inhibition of growth in this series of experiments. Inhibition by the other phorbol esters and related derivatives ranged from 46 % to 65%. In contrast, maximal inhibition in response to bryostatin 1 treatment was only 20 %. While none of the phorbol esters or related derivatives resembled bryostatin 1 in this system for inhibition of proliferation, it should be noted that the limited differences observed reflected the pattern of selectivity seen with the LNCaP cells. Thus, phorbol 12,13-dibenzoate and sapintoxin D induced a little less inhibition than the other compounds or PMA; indolactam V induced a little more inhibition. These differences, while small, were statistically significant ($p < 0.03$).

Attachment to the substratum is a second response of the U937 cells which differs between PMA and bryostatin 1. PMA induces attachment whereas bryostatin 1 has little effect. For the series of phorbol esters and related derivatives examined, the overall pattern resembles that seen for inhibition of proliferation in this cell type (Figure 6A, Table 1). All of the compounds induced attachment, with maximal levels of the total cells becoming attached ranging from 53% to 76%. In contrast, bryostatin 1 induced only 9%. More diversity is

observed in the dose response relationships. Dose response curves for some of the compounds were markedly biphasic, whereas in other cases little suppression was seen for doses above those inducing a maximal response. Again, sapintoxin D, phorbol 12,13-dibenzoate, and phorbol 12,13-dihexanoate showed the most suppression at high doses. Indolactam V, octyl-indolactam V, and ingenol 3-angelate showed the least suppression. In parallel with the effect of bryostatin 1, combination of a suppressing concentration of the phorbol derivative together with PMA largely resembled the effect of the suppressing concentration of the phorbol derivative alone (Figure 6B). Just as bryostatin 1 showed much less induction of attachment, it correspondingly was much more effective at blocking attachment in combination.

To determine whether we could detect any unique pattern of membrane interaction in the case of those derivatives that showed a lesser extent of PMA-like activity in any of the biological assays, we assessed their ability to insert into artificial membranes (Supplementary Figure 1). We have previously described the use of mixed membranes of dimyristoylphosphatidylcholine into which polydiacetylene (PDA) patches are incorporated. Interaction of lipophilic molecules with the membranes induces fluorescent signals from the PDA patches,^[25] and this technique has proven useful for evaluating the interaction of DAG-lactones with membranes.^[26] In the lipid/PDA vesicle system, the extent of membrane interaction corresponded to the lipophilicity of the molecules, thus suggesting that the uniqueness of the compounds which were less PMA-like could not be attributed to distinct membrane interactions, at least as can be detected by this method.

Discussion

Bryostatin 1 stands apart from PMA in the very different pattern of biological responses that it induces. In contrast, it has long been regarded that the various phorbol esters have generally similar biological effects, albeit they may differ substantially in potencies.^[27] Nonetheless, at the whole animal level, there are limited examples of different phorbol esters and related derivatives causing divergent responses. Most dramatically, the relatively hydrophilic (less lipophilic) derivatives 12-deoxyphorbol 13-acetate (prostratin) and 12-deoxyphorbol 13-phenylacetate are inhibitors of tumor promotion, whereas PMA is tumor promoting,^[16] and, in addition, these compounds inhibit some range of the other PMA induced responses in mouse skin.^[28] Likewise, in mouse skin phorbol derivatives with unsaturated side chains tend to be inflammatory but not tumor promoting, whereas their saturated derivatives are both inflammatory and tumor promoting.^[29] Such differences, of course, could reflect issues of drug absorption and stability in the whole animal.

Recent advances have suggested, however, that the differences in the patterns of biological response to phorbol esters and other PKC activators may be more profound. For example, analysis of combinatorial libraries of DAG-lactones, potent synthetic PKC ligands, indicated marked diversity in selectivity for different cellular assays.^[30] Likewise, it is abundantly clear that different phorbol esters and related derivatives may have different selectivity in cells for different isoforms.^[19, 31] Since different PKC isoforms have different effects, differences in structure activity relations would be predicted to lead to different patterns of cellular response.^[2] Moreover, it is now recognized that the PKC family of isoforms is only one of seven families of proteins with C1 domains that recognize diacylglycerol, phorbol esters, and related derivatives.^[2] Further, we have described ligands with selectivity for RasGRP3 compared to PKC isoforms.^[31-32] Such diversity suggests that PKC ligands may afford a richer fund of therapeutic biomolecules than had initially been recognized.^[7] Indeed, ingenol 3-angelate (PEP005), isolated from herbal preparations of *Euphorbia peplus*, is currently in phase III clinical trials (www.clinicaltrials.gov) for actinic keratosis and basal cell carcinoma.

Our findings provide unambiguous answers to the two issues motivating this study. First, can the difference in pattern of membrane localization of PKC delta induced by PMA and bryostatin 1 account for the different patterns of response in the LNCaP cells? Clearly it cannot, at the level of resolution provided by the GFP-PKC delta constructs and by differences in lipophilicity. This is important, because it argues that the lack of plasma membrane localization is not a robust measure of bryostatin-like activity, as it has sometimes been used.^[33] On the other hand, our findings should not be overinterpreted. Our results do not address whether localization at a higher level of resolution might be relevant or whether localization in combination with other factors might be determinative.

The second issue is whether the special pattern of biological activity of bryostatin 1 is unique to the bryostatin structural template or whether it may be more generalizable. Our findings indicate that, at least in the LNCaP system, the difference between bryostatin 1 and phorbol esters is not so great. In contrast, in the U937 leukemia cells, the difference is appreciably larger. We conclude that the U937 cell system provides a more stringent test of the homologous behavior of other ligands with bryostatin 1. A further conclusion is that the difference between bryostatin 1 and the phorbol derivatives examined here seems more quantitative than qualitative. The assays can be ranked in the following order of decreasing stringency: inhibition of proliferation of the U937 cells, induction of attachment of the U937 cells, inhibition of proliferation of the LNCaP cells, and induction of TNF α secretion in the LNCaP cells. The inhibition of proliferation of the U937 cells is the most demanding for homology; the induction of TNF α secretion is the least demanding. This continuum in behavior implies that the underlying mechanisms, at least for these responses that differentiate PMA and bryostatin 1 in these cell types, will be differences in degree of action.

It is important that the great sophistication demanded in the design and synthesis of novel bryostatin 1 analogs targeted for cancer chemotherapy is complemented by appropriate insights into the biological systems for testing these analogs. Our studies provide guidance in this effort.

Experimental Section

Materials

All phorbol ester derivatives were purchased from LC Laboratories (Woburn, MA). Sapintoxin D was from Enzo Life Sciences International Inc. (Plymouth Meeting, PA). The bryostatin 1 was provided by the Developmental Therapeutics Program, NCI (Frederick, MD). The LNCaP human prostate cancer cell line, the U937 human monocytic cell line, fetal bovine serum (FBS) and RPMI-1640 medium were from ATCC (Manassas, VA).

Growth of LNCaP cells

The growth of LNCaP cells was expressed as the difference in cell confluency before treatment and 72 hours after treatment. The confluency of LNCaP cells was followed in real time using an Incucyte instrument (Essen Instruments, Ann Arbor, MI). LNCaP cells were plated onto 48 well plates (25,000 cells /well) and 48 hours later were treated with the indicated compounds at different concentrations (final DMSO concentration 0.1% for each treatment). Phase contrast images of the cells were taken every 2-4 hours by the instrument for 4 days. The confluency of the cells was calculated by the instrument's program. The difference in confluency between 0 and 72 hours were calculated for each compound, subtracted from the difference for the DMSO control, and normalized to the maximal PMA response (10 nM).

Measurement of TNF-alpha

180,000 cells/well were plated on 24 well plates and 24 hours later were treated with the indicated drugs. The supernatants were collected 24 hours after treatment; they were centrifuged (4000 RPM for 5 min in an Eppendorf micro-centrifuge) to remove any cells and TNF-alpha levels were measured by ELISA following the manufacturer's instructions (Invitrogen, Carlsbad, CA).

Translocation of GFP-Tagged PKC Isoforms in LNCaP cells

Translocation of PKC-delta was evaluated as described before^[13] with some modifications. 60,000 LNCaP cells were plated on Ibi-treated dishes (Ibidi LLC, Verona, WI) and 48 hrs later were transfected with GFP-tagged PKC δ using Lipofectamine reagent in combination with Plus reagent following the recommendations of the manufacturer (Invitrogen, Carlsbad, CA). 24 hours later the cells were treated with the indicated drugs in confocal medium (DMEM without phenol red supplemented with 1% FBS) and time-lapse images were collected every 30 s using the Zeiss AIM software. The cells were examined in a Zeiss LSM 510 confocal microscopy system (Carl Zeiss Inc, Thornwood, NY) with an Axiovert 100M inverted microscope operating with a 25 mW argon laser tuned to 488 nm. Cells were imaged with a 63 $\times$ 1.4 NA Zeiss Plan-Apochromat oil immersion objective and with varying zooms (1.4 to 2).

The growth and attachment of U937 cells were measured as described earlier.^[8a] U937 cells were plated in 35 mm dishes at a density of 1×10^5 living cells ml⁻¹ and 24 hours later were treated with different concentrations of the drugs or DMSO (diluent concentration in each sample was 0.1%). After 72 hours, the number of non-attached cells present in the supernatant and the number of attached cells (determined after trypsinization) were counted using a Beckman particle counter (Beckman Coulter Inc., Fullerton, CA). The number of attached cells is expressed as percent of total cells.

Dose response curves and statistical analysis

Concentrations of compounds used in the different biological assays and their logP values are listed in Supplementary Table 1. Values listed in the text, unless otherwise noted, represent the mean $\pm$ SEM from at least three independent experiments. Statistical significance was determined using the Student's two-tailed t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The tested PKC ligands differed in both their structural templates and patterns of substitution

The chemical structures of the different phorbol esters, indolactams, ingenol 3-angelate and bryostatin 1.

Figure 2. PKC ligands vary greatly in the extent of inhibition of LNCaP cell growth and the degree to which their dose response curves are biphasic

LNCaP cells were treated for 72 hours with different concentrations (increasing doses from left to right with doses in half-log increments for all effective doses; details of concentrations in Supplementary Table 1) of the indicated compounds. Compounds are aligned based on their lipophilicity (LogP values) as indicated on the top panel. The change in confluency was detected using an Incucyte instrument and inhibition of growth normalized to that by 10 nM PMA as described in Materials and Methods. Values represent the mean $\pm$ SEM of at least four independent experiments ($n = 4$ to 9).

Figure 3. PKC ligands vary greatly in the extent of induction of TNF α secretion by LNCaP cells and the degree to which their dose response curves are biphasic

LNCaP cells were treated for 24 hours with different concentrations of the indicated compounds (aligned based on their lipophilicity, increasing doses from left to right with doses in half-log increments for all effective doses; see details of treatment in Supplementary Table 1) and TNF α secreted into the supernatant was measured by ELISA. Data were normalized to the maximal response induced by PMA (10 nM). Values represent the mean $\pm$ SEM of at least three independent experiments (n = 3 to 9).

Figure 4. The most lipophilic PKC ligands induce translocation of GFP-PKC delta mostly to plasma membrane while the less lipophilic PKC ligands, including bryostatin 1, induce translocation mostly to internal membranes

(LogP values of compounds are included in parentheses) LNCaP cells plated on ibi-treated dishes for better attachment were transfected with GFP-PKC delta and 24 hours later were treated with the indicated drugs. The translocation of GFP-PKC delta was detected by confocal microscopy in real time with images taken every 30 sec. Images taken at 0, 2, and 20 min are shown. They were representative of those from three independently performed experiments. Three representative series of images from eight independent experiments are shown for bryostatin 1, illustrating the range of variability observed. Boxed regions of the images taken at 20 min after treatment are enlarged for better visualization.

Figure 5. All tested PKC ligands except for bryostatin 1 induce inhibition of U937 cell growth
U937 cells were treated with different concentrations (increasing concentrations from left to right in log increments; see details in table 1) of the indicated compounds for 72 hours. The number of cells (attached and non-attached) were counted and graphed as percent of DMSO-treated control cells. The bars and error bars represent the mean and the standard error of the mean of at least three independent experiments (n = 3 to 9).

Figure 6. PKC ligands except for bryostatin 1 induce differentiation of U937 cells as indicated by attachment to the substratum and some show a biphasic response. Those PKC ligands which at high doses suppress cell attachment also, at these doses, antagonize the effect of PMA, as does bryostatin 1

A) U937 cells were treated with different concentrations (increasing concentrations from left to right in log increments; see details in Supplementary Table 1) of the indicated compounds for 72 hours. **B)** U937 cells were treated with 10 nM PMA in combination with 1000 nM of the indicated compounds (10,000 nM for prostratin, phorbol diacetate, indolactam V). The numbers of attached cells and total cells were counted and the attached cells were graphed as percent of total cells. The bars and error bars represent the mean and the standard error of the mean of at least three independent experiments (n = 3 to 7).

Table 1
Maximal responses and EC₅₀/IC₅₀ values of different PKC ligands for different biological end points

BIOLOGICAL RESPONSE	Growth of LNCaP cells		TNF α secretion from LNCaP cells		Growth of U937 cells		Attachment of U937 cells	
	Maximal inhibition (% of PMA response)	IC ₅₀ (nM)[a]	Maximal secretion (% of maximal PMA response)	EC ₅₀ (nM)[a]	IC ₅₀ (nM)[a]	EC ₅₀ (nM)[a]	Mean $\pm$ SEM	Mean $\pm$ SEM
PHORBOL ESTERS								
Prostratin	Mean $\pm$ SEM 45.9 $\pm$ 5.8	Mean $\pm$ SEM 278 $\pm$ 23	Mean $\pm$ SEM 58.8 $\pm$ 6.0	Mean $\pm$ SEM 720 $\pm$ 130	Mean $\pm$ SEM 1130 $\pm$ 340	Mean $\pm$ SEM 1730 $\pm$ 410		
Phorbol diacetate	58.1 $\pm$ 9.8	486 $\pm$ 86	62.7 $\pm$ 4.3	1990 $\pm$ 330	1450 $\pm$ 120	2750 $\pm$ 790		
Phorbol dibutyrate	65.0 $\pm$ 3.7	7.03 $\pm$ 0.41	52.6 $\pm$ 5.8	13.9 $\pm$ 2.4	5.5 $\pm$ 2.3	15.1 $\pm$ 2.5		
Indolactam V	123 $\pm$ 16	64.17 $\pm$ 13	84 $\pm$ 12	236 $\pm$ 34	337 $\pm$ 44	400 $\pm$ 100		
Sapintoxin D	44.6 $\pm$ 5.9	2.53 $\pm$ 0.25	53.4 $\pm$ 2.9	26.0 $\pm$ 8.7	2.95 $\pm$ 0.32	3.96 $\pm$ 0.39		
Phorbol dibenzoate	33.84 $\pm$ 0.78	11.8 $\pm$ 5.0	23.5 $\pm$ 1.2	29.0 $\pm$ 3.7	5.88 $\pm$ 0.90	12.1 $\pm$ 3.0		
Phorbol 13-decanoate	91.9 $\pm$ 6.9	109 $\pm$ 11	68.3 $\pm$ 4.6	298 $\pm$ 62	176 $\pm$ 33	240 $\pm$ 120		
Ingenol 3-angelate	102.2 $\pm$ 4.3	9.0 $\pm$ 2.0	100 $\pm$ 11	15.5 $\pm$ 3.6	6.0 $\pm$ 2.1	17.66 $\pm$ 0.81		
Phorbol dihexanoate	49.6 $\pm$ 4.2	1.74 $\pm$ 0.51	39.8 $\pm$ 2.6	5.9 $\pm$ 1.6	1.18 $\pm$ 0.11	4.0 $\pm$ 1.5		
Phorbol 12-myristate 13-acetate	100	1.56 $\pm$ 0.16	100	3.16 $\pm$ 0.27	0.36 $\pm$ 0.05	0.74 $\pm$ 0.19		
Octyl-indolactam V	115.4 $\pm$ 4.4	2.96 $\pm$ 0.36	96.9 $\pm$ 6.4	13.97 $\pm$ 0.29	7.93 $\pm$ 0.82	19.1 $\pm$ 4.4		
Phorbol didecanoate	71 $\pm$ 11	5.6 $\pm$ 1.4	87.9 $\pm$ 7.1	10.3 $\pm$ 2.1	1.11 $\pm$ 0.37	4.5 $\pm$ 1.2		
Bryostatins 1	19.6 $\pm$ 5.0	1.95 $\pm$ 0.53	1.50 $\pm$ 0.18	n.d.	n.d.	0.32 $\pm$ 0.14		

IC₅₀: effective inhibitory concentrations EC₅₀: effective concentrations

n.d. not determined because of low signal intensity or very few point for the first phase of the biphasic curve

[a] IC₅₀sub> and EC₅₀ were calculated based on 50% of the maximal response for the specific ligand. The values would be modestly different if another basis were used for the calculations but, since we do not know the mechanism accounting for the biphasic curves, we do not know if another basis would be preferable.