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Synthesis and Biological Evaluation of Fluorescent Bryostatin Analogues

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Abstract

To investigate the cellular distribution of tumor-promoting vs. non-tumor-promoting bryostatin analogues, we synthesized fluorescently labeled variants of two bryostatin derivatives that have previously shown either phorbol ester-like or bryostatin-like biological activity in U937 leukemia cells. These new fluorescent analogues both displayed high affinity for protein kinase C (PKC) binding and retained the basic properties of the parent unlabeled compounds in U937 assays. The fluorescent compounds showed similar patterns of intracellular distribution in cells, however; this argues against an existing hypothesis that various patterns of intracellular distribution are responsible for differences in biological activity. Upon further characterization, the fluorescent compounds revealed a slow rate of cellular uptake; correspondingly, they showed reduced activity for cellular responses that were only transient upon treatment with phorbol ester or bryostatin 1.

Keywords

bryostatin; fluorescent probes; Merle; protein kinase C; pyran annulation

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Conflict of Interest

The authors declare no conflict of interest.

Introduction

1,2-Diacylglycerols (DAG) are important secondary messengers that regulate numerous cellular functions such as apoptosis, differentiation, cell growth, and tumor promotion. Among the best-characterized mediators of DAG signaling are the family of protein kinase C (PKC) isoforms, which play important roles in cell proliferation, differentiation, and apoptosis^[1] and afford interesting targets for the treatment of a variety of diseases including cancer,^[2] human immunodeficiency virus HIV,^[3] diabetes,^[4] and Alzheimer's disease.^[5] Ligand binding drives a conformational change of PKC, leading both to enzymatic activation and to membrane association.^[6] Membrane association, in turn, influences enzyme specificity by controlling proximity to the different target proteins present in different subcellular locations.^[7] In addition to DAG, numerous potent exogenous ligands have been identified (Scheme 1), such as the phorbol and ingenol esters (tetracyclic diterpenes), aplysiatoxins (polyacetates), teleocidins (indolactams), and the bryostatins (macro-cyclic lactones).^[8] These ligands all function by inserting into a hydrophilic cleft on the C1 domain of PKC, thus completing a hydrophobic surface as well as contributing new hydrophobic or hydrophilic elements, depending on the ligand.^[9] Binding of a ligand to this C1 domain promotes membrane insertion and activation of the enzyme.

Although various ligands share this mechanism of PKC activation, the complicated downstream signaling pathways of the family of PKC isoforms, and of the related classes of signaling proteins with homologous C1 domains (e.g., RasGRPs, chimaerins, PKDs), result in different ligands having dramatically different and even contrasting biological outcomes. For example, although phorbol 12-myristate 13-acetate (PMA; Scheme 1) is the archetypical skin tumor promoter,^[10] 12-deoxyphorbol 13-acetate (prostratin) is not tumor-promoting.^[11] Bryostatin 1 has attracted particular attention because it is unique among C1 domain ligands. Not only does bryostatin 1 fail to induce most of the typical cellular responses that are induced by PMA, but it also blocks biological responses that it does not induce.^[8] PMA inhibits cell growth and induces attachment of U937 leukemia cells and inhibits the growth of the human LNCaP prostate cancer cell line. Bryostatin 1 has little effect on any of these endpoints but, when co-administered with PMA, will antagonize the response to PMA. Our group has utilized the U937 and LNCaP systems in an effort to link structural features of bryostatin 1 and closely related analogues to this unique pattern of bryostatin-like biology.^[12] Because the molecule is non-tumor-promoting^[13] and acts, in many instances, as an antagonist of PKC activity, bryostatin 1 has attracted substantial interest as a therapeutic candidate (> 80 phase I and phase II trials).^[14]

Bryostatin 1 shows multiple mechanistic differences compared to PMA. For some responses, it shows transient activity compared to PMA, followed by suppression of response.^[16] In multiple cell types, it displays a biphasic dose-response curve for downregulation of PKC δ , with partial protection from downregulation at higher bryostatin 1 concentrations,^[17] whereas PMA induces monophasic downregulation. Another difference that has been studied in detail is the effect on translocation. PMA causes PKC δ to initially translocate to the plasma membrane, followed by partial translocation to the internal membranes and the nuclear membrane. Contrastingly, bryostatin 1 causes PKC δ to translocate to the internal membranes and the nuclear membrane but not to the plasma membrane.^[18] Finally, whereas

PMA induces phosphorylation of PKC δ at Y311, among other sites, bryostatin 1 has little effect on phosphorylation at this site.^[12j]

Each of these mechanistic differences could contribute to a different pattern of biological response, depending on the specific cell type and the specific biological endpoint. A critical tool for dissecting the relative roles of these different mechanistic features has been the recent advances in bryostatin chemistry, now making available analogues that can permit correlation between retention or loss of a particular mechanistic feature and downstream biological responses.^[19] Our current view is that transient response is the predominant mechanism responsible for the differential effects of bryostatin 1 and PMA in U937 and LNCaP cell systems.^[12i] Although the pattern of translocation of PKC δ correlates well with the reported tumor-promoting ability of compounds in whole animal studies, its contribution to the unique behavior of bryostatin 1 as an antagonist of typical PMA responses remains unresolved.

We have previously shown that ligand lipophilicity is an important determinant for the pattern of PKC δ translocation and that the less lipophilic ligands, like bryostatin 1, tend to cause translocation of PKC δ to internal membranes and the nuclear membrane and also tend to be non-tumor-promoting.^[20] Moreover, using a series of fluorescent phorbol esters, we showed that compounds with long lipophilic hydrocarbon chains first accumulated in the plasma membrane before slowly equilibrating with internal membranes.^[21] Recruitment of PKC to a plasma-membrane-associated ligand afforded a plausible mechanism for localized PKC activation at the membrane, leading to phosphorylation of specific substrates. Likewise, localized activation of PKC at internal membranes might lead to phosphorylation of different substrates. We have also shown that, for a series of phorbol esters and related analogues of varying lipophilicities, some of the less lipophilic compounds had a diminished ability to inhibit LNCaP growth or to induce tumor necrosis factor α (TNF α) in the LNCaP cells compared to PMA; these effects were like those of bryostatin 1, albeit of considerably lesser magnitude.^[22] Bryostatin 1, however, is still unique in its ability to antagonize the effects of other C1 ligands, and, unlike bryostatin 1, all of the phorbol esters tested inhibited U937 cell growth comparably, although a few caused less cellular attachment.^[22] Although the cellular distribution of phorbol esters^[23] and indole alkaloids^[24] has been studied, little is known about the distribution of the much less lipophilic bryostatins and analogues.

Our goal in the present study was to assess whether differences in cellular distribution could explain the different biological profiles of a pair of bryostatin derivatives, one PMA-like and the other bryostatin-like, in U937 cells. As the addition of a fluorescent tag modified the structure of the parent compounds as expected, a related objective was to characterize the patterns of biological response of the compounds.

Results and Discussion

In order to identify a suitable attachment site for a fluorescent dye, we utilized existing bryostatin structure-function relationships. Contributions by several research groups have led to the synthesis of over 100 bryostatin analogues, providing a detailed understanding of the functional role for many bryostatin substituents.^[12b-f,25] Our group, specifically, has

prepared more than 20 analogues, many of which rival the potency of bryostatin 1 itself (Scheme 2).^[12d] For example, one compound, designated Merle 28 and lacking the B ring methyl ester, was shown to have a binding affinity of (0.52 ± 0.06) nM for PKC α , whereas the affinity of bryostatin 1 in comparable assays was (0.73 ± 0.05) nM. Early analogue work established that binding of bryostatin, or a bryopyran analogue, to PKC is primarily mediated through functionality on the C ring (southern portion) of the molecule,^[25c] although the A and B rings (northern portion) also contribute.^[12g] The A and B rings make their primary contribution by influencing the pattern of biological responses, provided that the compound possesses sufficient PKC binding affinity. As described above, tumor-promoting ligands such as PMA inhibit proliferation and induce attachment in U937 leukemia cells, whereas bryostatin 1 has little effect on either response. However, when co-administered, bryostatin 1 blocks the effects of PMA in a dose-dependent manner. We have described analogues as either PMA-like or bryostatin-like, based on their behavior in these assays. Merle 23^[12d] and Merle 27^[12c] were shown to be PMA-like, whereas Merle 28^[12b] and Merle 30^[12e] were bryostatin-like. The most notable difference between these analogues is the number of polar functional groups displayed on the A and B rings. Both Merle 28 and Merle 30 contain two oxygen substituents each; Merle 23 and Merle 27 possess none and one, respectively. As our current binding model has the A and B rings forming a cap on the top of the C1 domain,^[12e] we hypothesized that differences in polar substituents on these rings influence membrane interaction and subcellular distribution of the ligands, with consequent effects on the downstream responses. To investigate this hypothesis, the PMA-like Merle 23 and the bryostatin-like Merle 28 were selected for further study; fluorescent versions of these bryostatin analogues were used to help us assess their membrane interactions.

Synthesis of Merle 45

Retrosynthetically, we anticipated that Merle 45 could be formed through a highly convergent coupling of fully functionalized A ring **2** and C ring **3** by using a pyran annulation and Yamaguchi esterification (Scheme 3). Based on previous experience, we anticipated that having the C9 ketal in place would be problematic, due to formation of a spiroketal byproduct under conditions typically used for intermolecular pyran annulation (TMS-OTf, Et₂O), so we decided to first connect the two pieces through a Yamaguchi esterification, followed by milder acid-mediated pyran annulation conditions.^[26,27] Based on an earlier reported docking structure of bryostatin in the C1 domain of PKC and structure-activity relationship (SAR) data for the C20 ester position, we reasoned that the C20 position would be well suited to addition of a bulky fluorophore.^[12d-e,28] The C20 ester, which is mostly solvent exposed and does not make crucial interactions with the protein, was previously shown to tolerate substantial variations in structure, including bulky substituents.^[23h] The fluorescent BODIPY FL tag was envisioned to be installed at a late stage by click chemistry between a BODIPY FL-containing azide^[29] and alkyne **1**.

The synthesis started from the hydrolysis of previously described thioester **4**^[30] by using NBS and water to give carboxylic acid **5** (Scheme 4).^[31] The β -hydroxyallylsilane was installed by using the Williams reaction between (*S,S*)-allylboronate **6** and aldehyde **5** to give a homoallylic alcohol, which was protected as the TES ether.^[32] This protected

allylsilane (**7**) was obtained in 77% yield as a 6:1 mixture of diastereomers over the two steps.

The southern portion of the bryostatin analogue was synthesized beginning from previously described C ring **3**.^[26] The acetate was removed by using $K_2CO_3/MeOH$, the resulting alcohol was esterified with 5-hexynoic acid, and the TBS ether was removed to give allylic alcohol **8**. The unmasked alcohol was oxidized, and the C25 PMB was deprotected to produce the free C25 alcohol. At this point, intramolecular pyran annulation was investigated (Scheme 5).

At first, the intramolecular pyran annulation did not work, even though a similar substrate was used during the synthesis of bryostatin **9** (Scheme 6).^[27] When compound **10** was subjected to PPTS in MeOH, only acetal **11** was formed, and no macrocycle was observed. On the other hand, after removal of the C19 methyl ketal and Yamaguchi esterification to form ester **12**, (Scheme 7) subjection to the same reaction conditions resulted in macrocyclization to give tricyclic macrolactone **13** in a 70% yield with no spirocyclic side product observed. Thus, the macrocyclization failed with a methoxy group at C19, but proceeded in good yield with a hydroxy group at this position. Dependence on the presence of the C19 hydroxy is not fully understood; however, in the crystal structure of bryostatin **1**,^[33] the C19 alcohol donates a hydrogen bond to the C3 alcohol, which is within hydrogen bonding distance of both the A and B ring pyran oxygens. This internal hydrogen bonding network is required for biological activity, and partial formation of this network might serve to orient the aldehyde and β -hydroxyallylsilane for pyran annulation. It thus seems highly likely that the intramolecular pyran annulation reaction is, in fact, a hydroxy-directed event.

The synthesis was continued by removing the protecting groups, with exposure of BPS ether **13** to HF-py, followed by a global deprotection with $LiBF_4$, to give alcohol **14** in a 66% yield over two steps. Finally, azide **15** (Supporting Information) was coupled with **14** in the presence of copper(I) iodide and iPr_2NEt to afford Merle 45 in 80% yield.

Synthesis of Merle 44

Using our pyran annulation methodology^[12d,34] to couple A ring **16**^[12d,30a] with fully functionalized C ring **17**,^[26] we were able to produce **18** in 92% yield (Scheme 8). Unlike the PPTS-mediated pyran annulation, TMSOTf-mediated cyclization does not require a C19 hydroxy group. As the top portion of Merle 44 has less functionality, the stronger TMSOTf can be used to construct the B ring with the C19 methoxy group still present. Closing the macrocycle began by first deprotecting the C1 BPS ether, followed by consecutive Parikh-Doering and Pinnick oxidations to give C1 carboxylic acid **19**. The C25 TBS ether was then removed, followed by a Yamaguchi macrolactonization to afford the desired tricyclic macrolactone (**20**). The C20 acetate was removed, and the resulting alcohol was esterified with 5-hexynoic acid to give the desired ester (**21**). The PMB ether was removed with DDQ, and global deprotection with $LiBF_4$ gave the bryopyran intermediate, which was coupled with azide **15** to afford Merle 44 in 80% yield.

Biological evaluation of Merle 44 and Merle 45

Consistent with the tolerance of the C20 position for bulky substitutions, the bulky fluorophore did not substantially affect the in vitro binding of Merle 44 and Merle 45 to PKC α . In a competition binding assay with [3 H]PDBu and purified PKC α , Merle 44 bound with a K_i value of 0.64 ± 0.08 nM ($n = 3$), which was very similar to that for Merle 23 ($K_i = 0.70 \pm 0.01$ nM). Merle 45 bound with a K_i value of 0.32 ± 0.03 nM ($n = 3$), which was also very similar to that of Merle 28 ($K_i = 0.52 \pm 0.06$ nM). These values were likewise similar to that for bryostatin 1 ($K_i = 0.73 \pm 0.05$ nM), showing that inclusion of the fluorescent side chain at the C20 position had very little effect on ligand binding to PKC α in vitro.

The motivation for the preparation of Merle 44 and Merle 45 was to prepare fluorescent analogues of Merle 23 and Merle 28, respectively. Merle 23 is largely PMA-like in its biological activity^[12d] in U937 cells, whereas Merle 28 is bryostatin-like.^[12b] A crucial issue was whether the fluorescent compounds retained the patterns of biological activity of their non-fluorescent congeners.

Merle 45, like Merle 28 and bryostatin 1, did not inhibit proliferation (Figure 1) or induce attachment (Figure 2) of U937 cells. Moreover, like Merle 28 and bryostatin 1, Merle 45 antagonized the effects of PMA when coadministered ($p = 0.1767$ for any difference between the effect of any of the three compounds). Interestingly, Merle 45 induced 7.1% less attachment than was seen with Merle 28 ($p = 0.0002$) and was a more potent antagonist of attachment induced by PMA, resulting in a pattern that appeared even more bryostatin-like than that of Merle 28 ($p = 0.8671$ for comparison of M45 with Bryo 1; $p = 0.0200$ for comparison of M28 with Bryo 1).

Just as the fluorescent Merle 45 preserved the bryostatin-like biology activity of Merle 28, fluorescent Merle 44 largely preserved the PMA-like biological behavior of Merle 23. Like Merle 23 and PMA, Merle 44 inhibited growth (Figure 1) and induced attachment (Figure 2) of the U937 cells, although it should be noted that the level of inhibition was 9.6% less than that achieved by the optimal dose of Merle 23 ($p = 0.0060$). Additionally, like Merle 23, Merle 44 did not antagonize the effects of PMA in U937 cells when co-administered ($p = 0.0034$ for comparison of M44 with Bryo 1; $p = 0.0016$ for comparison of M23 with Bryo 1).

Secretion of TNF α provided another differential response of the U937 cells to PMA and bryostatin 1, with bryostatin 1 inducing limited release compared to PMA.^[12j,35] Once again, Merle 45 behaved very similarly to bryostatin 1 and Merle 28 with regard to inducing TNF α release (Figure 3). Merle 44 more closely resembled PMA and Merle 23 in inducing a higher level of release, albeit lower than that induced by Merle 23. Interestingly, the maximal response was shifted to higher concentrations relative to Merle 23 by an order of magnitude. A similar shift was evident in the maximal doses of Merle 44 versus Merle 23 for inhibition of proliferation and for induction of attachment of the U937 cells. In all three biological assays, Merle 44 was two orders of magnitude less potent than Merle 45. This was in marked contrast to the in vitro binding assays, in which Merle 44 and Merle 45 were very similar, and illustrated that other factors, in addition to PKC binding affinity, contribute to biological efficacy.

Although the U937 cells provide a robust measure of the difference in biological response to PMA and bryostatin 1 (or its analogues)—as do several other cell lines, such as K562 and MV4-11^[12f]—Toledo cells, derived from a non-Hodgkin's leukemia, showed growth inhibition with both PMA and bryostatin 1. They thus provide a convenient and straightforward measure of intrinsic activity, and they also showed somewhat greater sensitivity to PMA and bryostatin 1 than did the U937 cells. In this system, Merle 44 once again stood out as being an order of magnitude less potent than Merle 23 (Figure 4). All other compounds were relatively close to one another in potency. IC₅₀ values were as follows: PMA (IC₅₀ = (0.57±0.27) nM), bryostatin 1 (IC₅₀ = (0.091±0.026) nM), Merle 28 (IC₅₀ = (0.111±0.009) nM), Merle 45 (IC₅₀ = (0.334±0.068) nM), Merle 23 (IC₅₀ = (0.33±0.11) nM), and Merle 44 (IC₅₀ = (3.17±0.67) nM).

Localization of Merle 44 and Merle 45 in LNCaP cells

We have previously described that different PKC ligands can drive translocation of multiple PKC isoforms to different sub-cellular locations. In particular, the tumor-promoting phorbol ester PMA induced initial localization of PKCδ to the plasma membrane, with subsequent relocalization to internal membranes, whereas non-promoting phorbol esters and bryostatin 1 caused the initial translocation of PKCδ to internal membranes.^[18] Fluorescently labeled Merle 44 and Merle 45 provided an opportunity to evaluate whether ligand localization could determine if a ligand would have bryostatin-like biology. The localization and rate of uptake of Merle 44 and Merle 45 were visualized in real time by confocal microscopy in LNCaP cells. These cells were used because they are attached cells, unlike U937, but, like U937 cells, have differential biological responses to PMA and bryostatin 1.^[12j] Both fluorescent compounds showed similar internal membrane distribution with no selective presence in the plasma membrane (Figure 5A). This contrasted with the initial plasma membrane described for PMA.^[18] We concluded that differential membrane localization of the ligand, with PMA-like behavior (as with Merle 44) reflecting early predominantly plasma membrane localization and bryostatin-like behavior (as with Merle 45) reflecting early internal localization, cannot account for the differential biological response. The pattern of ligand localization was consistent with our finding that initial plasma membrane localization of a ligand is characteristic of more lipophilic compounds (higher ClogP), and initial internal localization is characteristic of less lipophilic compounds.^[22] The calculated ClogP values of 3.71 for Merle 44 and of 4.68 for Merle 45 were higher than those of the parent compounds (Merle 23, ClogP = 2.67; Merle 28, ClogP = 1.70) but lower than that of PMA (ClogP = 6.65).

A further striking feature of the compounds was their slow rate of uptake. To better quantitate the rate of uptake, uptake of 100 nM Merle 44 and Merle 45 was determined to avoid saturation of the optical signal, and Cos7 cells were used rather than LNCaP cells, as Cos7 cells did not change their shape or show mobility on culture dishes after ligand addition (Figure 5B). Merle 45 showed modestly faster uptake than did Merle 44. Times for half-maximal uptake of 100 nM Merle 45 and Merle 44 into the Cos7 cells were (16.3±2.8) min and (22.8±1.6) min ((980±170) s and (1368±93)s), respectively. Such slow uptake will necessarily impose uncertainty in efforts to measure dose-response relations for early biological endpoints because the internal concentration of the ligand is changing. In

addition, to the degree that different PKC isoforms have different EC₅₀ values for stimulation, the gradient of rising ligand levels with time predicts that there will be sequential activation of those PKC isoforms, at least at lower concentrations of ligand. We have previously described that a series of DAG-indololactones, which also function as ligands for PKC, showed rapid uptake, with half-times within a range of 1 min.^[36] In contrast, the slow rate of uptake of Merle 44 and Merle 45 was consistent with the slow rates of uptake that we previously reported for several fluorescent phorbol ester derivatives.^[21] Finally, we can conclude that the limited difference in the patterns of substitution in the A and B rings of Merle 44 and Merle 45 (and, correspondingly, of Merle 23 and Merle 28) had only a modest effect on the rates of uptake.

Synthesis and biological evaluation of fluorescent phorbol esters

To provide fluorescent phorbol esters for comparison with the fluorescent Merle 44 and Merle 45, we prepared phorbol esters containing the same BODIPY tag, taking advantage of previous phorbol analogue chemistry.^[37] Starting from commercially available PMA, the primary alcohol was protected as a TBS ether, and the C12 and C13 esters were hydrolyzed to give tetrol **23**. The C13 alcohol could then be selectively acylated because of its placement on the convex face of this highly substituted ring system to give ester **24**.^[38] 5-Hexynoic acid was then used in a Yamaguchi esterification to install the alkyne moiety at the C12 position to construct **25**. The TBS ether was deprotected to expose the terminal alcohol on C20 by using HF-pyridine, and finally, a copper-catalyzed click cycloaddition was performed to furnish FPMA1 and FPMA2.^[39]

Unlike the bryostatin analogues, the K_i value of FPMA1 was greatly affected ($K_i = (201 \pm 24)$ nM vs. $K_i = 2.6$ nM for PMA) by the replacement of the 12-tetradecanoate ester of PMA with the fluorescent group and linker. Correspondingly, the potency of phorbol FPMA1 in the U937 and Toledo cell assays was dramatically (ca. 10 000-fold) reduced, with growth in the Toledo cells only beginning to show inhibition at 3000 nM and effects on the U937 cells only beginning to be induced at 10 000 nM, with possible toxicity at 30000 nM (Figure S1 in the Supporting Information). Based on these observations, FPMA1 appeared to be PMA-like, but its weak activity limited characterization. Localization of FPMA1 in LNCaP cells was also examined. Its localization was similar to that of Merle 44 and Merle 45, translocating mostly to internal membranes (data not shown).

Along with FPMA1 (Clog $P = 3.42$), we prepared the fluorescent derivative FPMA2 (Clog $P = 5.54$), in which we enhanced the lipophilicity by exchanging the C13 acetate for a longer aliphatic ester (Scheme 9). The K_i value of FPMA2 for PKC α was much improved ($K_i = (2.21 \pm 0.36)$ nM, $n = 4$).

FPMA2 inhibited U937 cell growth and induced attachment of U937 cells to a similar level but with different potency, as did PMA (Figure 6). The combination of FPMA2 (1000 nM) and PMA gave growth inhibition similar to that for PMA alone and induced attachment to a similar level as seen for PMA alone. At 10000 nM FPMA2, a little less attachment was seen for the combination than for PMA alone, suggestive of limited toxicity at this concentration and incubation time (60 h). Like PMA and bryostatin 1, FPMA2 inhibited Toledo cell

growth. In these cellular assays FPMA2 remained appreciably less potent than PMA, with EC₅₀ values between 100 and 1000 nM.

PKC translocation in LNCaP cells in response to treatment with Merle 44, Merle 45, and FPMA2

Merle 44 (Figure 7A), along with FPMA2 (Figure 7B), failed to induce clear translocation of PKC δ . As a positive control, 1 μ M PMA was added after 10 min incubation with 1 μ M FPMA2 and yielded the characteristic translocation of PKC δ to the plasma and nuclear membranes, as well as to internal membranes (Figure 7B). Merle 45 induced weak translocation of PKC δ at later times (Figure 7A) visible mostly in cells that showed nuclear PKC δ staining. For PKC ϵ , 1 μ M Merle 45 induced partial translocation to the plasma membrane. 3 μ M FPMA2 induced some translocation, although not as much as 1 μ M Merle 45. As with PKC δ , 1 μ M Merle 44 failed to induce translocation of PKC ϵ in this time range (Figure 7C).

PKC isozyme selectivity of Merle 44 and Merle 45

Although the specific goal of the development of Merle 44 and Merle 45 was to assess whether the ligands localized differently in the cells, while retaining PMA-like or bryostatin-like biological activity, we also further characterized these novel compounds. In U937 cells, the prominent isoforms of PKC are PKC β II, PKC δ , and PKC ϵ , whereas in LNCaP cells, they are PKC α , PKC δ , and PKC ϵ .^[40] We previously showed that in U937 cells, PKC β II and PKC δ are modulated by bryostatin 1 and PMA, whereas there was no downregulation of PKC ϵ when treated with these ligands.^[12i] Bryostatin 1 and similar analogues are unique in inducing a biphasic downregulation of PKC δ in these and other cells with maximum downregulation at 1 and 10 nM bryostatin 1 and partial protection from downregulation at higher bryostatin 1 concentrations. Here, we found that both PMA and the bryostatin-like compounds downregulated PKC β II at 24 h but with very different potencies (Figure 8A). For PKC δ downregulation (Figure 8B), Merle 45 did not protect PKC δ from downregulation at high concentrations unlike bryostatin 1 or Merle 28. Merle 23 and Merle 44 were less potent than Merle 45 for downregulation but again showed no protection of PKC δ downregulation at high doses. The differences between Merle 45 and bryostatin 1, as well as other Merle compounds emphasizes, as we have already described for Merle 23, that different derivatives can dissect different endpoints of behavior.^[12j]

Signal transduction induced by different compounds in LNCaP cells

We have shown that the fluorescent derivatives based on the bryostatin-like Merle 28 and the PMA-like Merle 23 retain the bryostatin-like or PMA-like biology of their parent compounds in U937 cells, although their intracellular localization is very similar in LNCaP cells, another cell line in which PMA and bryostatin 1 have markedly different effects on growth and other biological responses.^[22] We have also shown evidence for unusually slow uptake of the fluorescent compounds into the cells. This slow uptake might be predicted to influence the signal transduction events, especially the early phosphorylation events, induced by the compounds. We therefore evaluated a series of signal transduction responses, including phosphorylation of PKC δ , PKD1, MEK1/2, and ERK1/2 and changes in the levels of the early response proteins cFos and EGR1 in LNCaP cells treated for 30, 60, and 150

min. Whereas we have previously characterized differential effects of PMA and bryostatin 1 in both the U937 cells and LNCaP cells,^[12i] here we focused on the LNCaP cells because the effects of the compounds on gene expression were somewhat more marked.

Although we observed a complicated pattern of signaling responses for the different compounds, two clear conclusions emerged (Figure 9). The first clear conclusion was that PMA, bryostatin 1, Merle 45, and Merle 44 each had a different pattern of response, as shown by comparison of the patterns of phosphorylation of PKC δ Ser299, PKC δ , Tyr311, and PKD1 Ser744/Ser748. The second conclusion was that, for responses that were characterized by transiency, the responses to Merle 45 and Merle 44 never achieved the maximal levels achieved by PMA (Figure 9). The pattern of behavior fits with the model that the transient responses turn off before a fully stimulatory dose of the slowly penetrating ligand is achieved.

Conclusion

Installing the BODIPY FL tag at the C20 position did not have a substantial effect on the in vitro binding affinity of the new fluorescently labeled analogues, which largely retained the character of the parent compounds as PMA-like or bryostatin-like in their effects on U937 cell proliferation and attachment. Imaging revealed that the compounds that are PMA-like and bryostatin-like did not differ in intracellular localization, arguing that ligand localization cannot account for the different patterns of biological response. We previously described that structural variation can dissect different aspects of the biological response. This again was true for Merle 45, which retained its bryostatin-like behavior for U937 cell proliferation and attachment but now showed monophasic down regulation of PKC δ , in contrast to the biphasic down regulation in response to bryostatin 1. Likewise, Merle 45 and 44 each showed a unique pattern of downstream signaling events. Both Merle 45 and Merle 44 penetrated into cells slowly, over a time course of many minutes. Consistent with their slow penetration, Merle 45 and Merle 44 showed slower induction of some cellular responses and a lower level of stimulation for responses that were only transiently expressed. These differences in response of individual ligands emphasize once again the PMA-like or bryostatin-like represent a simplification and that abundant opportunities exist to match a specific pattern of response to a specific therapeutic application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

U937 cells were treated with the indicated concentrations of ligands alone or in the presence of 10 nM PMA. The cell number was counted at 60 h post treatment and expressed relative to a DMSO control. Values represent the mean and 95% confidence intervals (shaded region) from a linear regression model including at least four ($n = 4-6$) independent experiments per drug/concentration combination, with the exception of Merle 45 (3000 nM), which only had two observations. The ligand concentration when used in combination with PMA was 1000 nM except for Merle 44 when it was 3000 nM.

Figure 2.

U937 cells were treated with the indicated concentrations of ligands alone or with the indicated ligands in the presence of 10 nM PMA. Attachment was measured at 60 h of treatment. Values represent the mean and 95% confidence intervals (shaded region) from a log-linear regression model including at least four ($n = 4-6$) independent experiments per drug/concentration combination, with the exception of Merle 45 (3000 nM), which only had two observations. The ligand concentration when used in combination with PMA was 1000 nM except for Merle 44 when it was 3000 nM.

Figure 3. Secretion of TNF α . U937 cells were treated for 60 h with the indicated concentrations of the ligands. In each experiment, the level of secreted TNF α under each treatment condition was normalized to the level of secreted TNF α for the cells treated with 100 nM PMA. Values represent the mean of at least four ($n = 4-6$) independent experiments. Bars show standard error of the mean.

Figure 4.

Growth of Toledo cells. Number of Toledo cells was determined after treatment with the indicated concentrations of the ligands for 72 h and expressed as a percentage of the control. Values represent the mean of at least three independent experiments ($n = 3-5$). Bars show standard error of the mean.

Figure 5.

Localization of Merle 44 and Merle 45 in LNCaP cells and their uptake into Cos7 cells. A) LNCaP cells were transfected with mCherry PKC δ and imaged after treatment for the indicated times with 1000 nM of Merle 44 or Merle 45. Results are from one representative experiment (five independent experiments were performed under these conditions). B) Uptake of Merle 44 (●) and Merle 45 (●) into Cos7 cells as a function of time. Cos7 cells were transfected with mCherry PKC δ or mCherry PKC ϵ and imaged after treatment for the indicated times with 100 nM Merle 44 or Merle 45. Values represent the fluorescent

intensity of the whole field of view normalized to the maximal signal of each individual experiment and represent the mean of 7–8 experiments. Bars show standard error of the mean. The arrow indicates addition of the drug.

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Figure 6.

Biological analysis of FPMA2. A) Growth and B) attachment of U937 cells after treatment for 60 h with the indicated concentrations of PMA, bryostatin 1, and FPMA2. C) Inhibition of growth of Toledo cells after treatment for 72 h. All values represent the mean $\pm$ S.E.M. of at least five independent experiments ($n = 5-7$). D) Comparison of structures of PMA and FPMA2.

Figure 7.

Imaging of mCherry-tagged PKC δ (A, B) and PKC ϵ (C) in LNCaP cells after treatment with Merle 44 and Merle 45 (A, C) or FPMA2 (B, C). The red signal represents mCherry; the green signal represents BODIPY FL-labeled ligands. Results are representative of 3–6 independent experiments. C) Imaging of mCherry-tagged PKC ϵ in LNCaP cells after treatment with Merle 44, Merle 45, or FPMA2. Two representative sets of images from 3–6 independent experiments are shown. The red signal represents mCherry.

Figure 8.

Downregulation of A) PKCβII and B) PKCδ in U937 cells after treatment for 24 h with the indicated concentrations of compounds. Values represent the mean of at least four independent experiments ($n = 4-7$). Bars show standard error of the mean. Values represent signal intensity (AU) normalized to the DMSO control (100%).

Figure 9.

A)-C), E)-H) Signal transduction in LNCaP cells treated for the indicated times with DMSO (●), PMA (●), Bryo 1 (●), Merle 28 (●), Merle 45 (●), Merle 23 (●), Merle 44 (●), FPMA2 (●), and PDBu (●). All compounds were used at a concentration of 1000 nM except for FPMA2, which was used at a concentration of 3000 nM. Western blot signals were normalized to the 60 min signal induced by 1000 nM PMA. All values represent the mean $\pm$ S.E.M. of at least three independent experiments ($n = 3-6$). D) The average signal intensity for phosphorylation of PKCδ at Ser299 and Tyr311 and phosphorylation of PKD1 at

Ser744/748 after 30 min treatment with PMA, Bryo 1, Merle 44 and Merle 45 (1000 nM). Responses are normalized to those for PMA at 60 min.

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Scheme 1.

Comparison of structurally distinct PKC ligands. The binding affinities reported here were measured by competitive binding assays with a mixture of PKC isozymes.^[15]

Merle 28 (**1**): $R^1 = H$, $R^2 = OH$; $K_i = 0.52$ nM; Bryo-like
Merle 30: $R^1 = CO_2Me$, $R^2 = H$; $K_i = 0.38$ nM; Bryo-like

Merle 27: $K_i = 3.0$ nM; PMA-like

Merle 23 (**2**): $K_i = 0.70$ nM; PMA-like

Scheme 2.

Side-by-side comparison of synthetic bryostatin analogues. The binding affinities reported here were measured by competitive binding assays with purified PKC α .^[12b-e]

Scheme 3.

Retrosynthetic analysis of Merle 44 and Merle 45.

Scheme 4.

Synthesis of the A ring portion of Merle 45. a) NBS, THF/ H_2O (87%); b) **6**, DTBMP, CH_2Cl_2 , -78°C (77%, 6:1 *dr*); c) TESCl, im., CH_2Cl_2 , 0°C (90%).

Scheme 5.

Synthesis of the C ring portion of Merle 45. a) K_2CO_3 , MeOH, 3 h; b) hex-5-ynoic acid, 2,4,6- Cl_3PhCOCl , DMAP, Et_3N , toluene (80% over 2 steps); c) HF·Py, THF (93%); d) MnO_2 , benzene; e) DDQ, H_2O , CH_2Cl_2 , (77% over 2 steps).

Scheme 6.

Attempt to cyclize by using an intramolecular pyran annulation reaction. a) PPTS, MeOH (70%).

Scheme 7.

Synthesis of Merle 45. a) HF/H₂O, MeCN; b) 2,4,6-Cl₃PhCOCl, **7**, Et₃N, THF; then alcohol, DMAP, toluene, (70% over 2 steps); c) PPTS, MeOH (70%); d) HF-Py, THF; e) LiBF₄, CH₃CN/H₂O, 80°C (66% over 2 steps); f) **15**, CuI, *i*Pr₂NEt, THF (80%).

Scheme 8.

Synthesis of Merle 44. a) TMSOTf, Et₂O (92%); b) TBAF, AcOH, DMF (75%); c) SO₃-Py, *i*Pr₂NEt, DMSO, CH₂Cl₂; d) 2-methylbut-2-ene, *t*BuOH, KH₂PO₄, NaClO₂ (90% over 2 steps); e) HF·Py, THF; f) 2,4,6-Cl₃PhCOCl, Et₃N, THF; then DMAP, toluene (70% over 2 steps); g) K₂CO₃, MeOH, 1 h; h) hex-5-ynoic acid, 2,4,6-Cl₃PhCOCl, DMAP, Et₃N, toluene (70% over 2 steps); i) DDQ, H₂O, CH₂Cl₂; j) LiBF₄, CH₃CN/H₂O, 80°C (70% over 2 steps); k) **15**, CuI, *i*Pr₂NEt, THF (80%).

Scheme 9.

Synthesis of FPMA1 and FPMA2. a) TBSCl, DMAP, imidazole, DMF (95%); b) KCN, MeOH; c) (RCO₂)₂O, Et₃N, CH₂Cl₂/THF (1:1) (R = Me: 64%, R = (CH₂)₄Me: 78% over 2 steps); d) hex-5-ynoic acid, 2,4,6-trichlorobenzoyl chloride, DMAP, Et₃N, PhMe (R = Me: 75%, R = (CH₂)₄Me: 83%); e) HF·py, THF; f) **15**, CuI, *i*Pr₂NEt, THF, 70°C, 12 h (R = Me (FPMA1): 51%, R = (CH₂)₄Me (FPMA2): 85% over 2 steps).