Biochemical Pharmacology 81 (2011) 1296–1308

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The synthetic bryostatin analog Merle 23 dissects distinct mechanisms of bryostatin activity in the LNCaP human prostate cancer cell line

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ARTICLE INFO

Article history:
Received 19 January 2011
Accepted 22 March 2011
Available online 30 March 2011

Keywords:
Protein kinase C
Phorbol ester
Cell signaling
Bryostatin analog

ABSTRACT

Bryostatin 1 has attracted considerable attention both as a cancer chemotherapeutic agent and for its unique activity. Although it functions, like phorbol esters, as a potent protein kinase C (PKC) activator, it paradoxically antagonizes many phorbol ester responses in cells. Because of its complex structure, little is known of its structure-function relations. Merle 23 is a synthetic derivative, differing from bryostatin 1 at only four positions. However, in U-937 human leukemia cells, Merle 23 behaves like a phorbol ester and not like bryostatin 1. Here, we characterize the behavior of Merle 23 in the human prostate cancer cell line LNCaP. In this system, bryostatin 1 and phorbol ester have contrasting activities, with the phorbol ester but not bryostatin 1 blocking cell proliferation or tumor necrosis factor alpha secretion, among other responses. We show that Merle 23 displays a highly complex pattern of activity in this system. Depending on the specific biological response or mechanistic change, it was bryostatin-like, phorbol ester-like, intermediate in its behavior, or more effective than either. The pattern of response, moreover, varied depending on the conditions. We conclude that the newly emerging bryostatin derivatives such as Merle 23 provide powerful tools to dissect subsets of bryostatin mechanism and response.

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1. Introduction

Protein kinase C (PKC) has emerged as an exciting therapeutic target, reflecting its central role in cellular signaling, its differential regulation in a range of cancers, and the identification of natural products or their derivatives targeted to PKC that have entered clinical trials [1]. The PKCs comprise a family of serine/threonine specific protein kinases, of which the classic PKC isoforms (alpha, betaI, betaII, and gamma) respond to diacylglycerol and calcium through their C1 and C2 domains, respectively, whereas the novel PKC isoforms (delta, theta, epsilon, and eta) respond only to diacylglycerol. Like most kinases, the PKCs are further regulated in a complex fashion by phosphorylation – by other serine/threonine and tyrosine specific protein kinases, by autophosphorylation, and by phosphorylation by other PKC isoforms. Diacylglycerol is a ubiquitous lipophilic second messenger, generated through the breakdown of phosphatidylinositol 4,5-bisphosphate consequent to activation of phospholipase C downstream of receptor tyrosine kinases and G-protein coupled receptors, as well as indirectly following activation of phospholipase D. Diacylglycerol recognition occurs through the C1 domains of PKC, which function as hydrophobic switches to bring about both PKC activation as well as the translocation of the PKC to membranes, enhancing its access to membrane bound substrates. Consistent with the ternary nature of the bound complex, which comprises ligand, C1 domain, and

Abbreviations: PKC, protein kinase C; DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated kinases; MEK, MAPK/ERK kinase 1; MAPK, mitogen-activated protein kinase; JNK, cJun N-terminal kinases; PMA, phorbol 12-myristate 13-acetate; HAART, highly active antiretroviral therapy; DAG, diacylglycerol; GFP, green fluorescent protein; TNF-alpha, tumor necrosis factor alpha; MARCKS, myristoylated alanine rich C kinase substrate; TACE, TNF-alpha converting enzyme; TRAIL, TNF-related apoptosis-inducing ligand.

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0006-2952/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.bcp.2011.03.018

cellular membrane, emerging evidence strongly argues for the role of membrane microdomains in contributing to ligand specificity [2].

In addition to diacylglycerol, a range of complex natural products of diverse structures have been identified which function as ultrapotent analogs of diacylglycerol, binding to the C1 domain. These include the phorbol esters (diterpenes), the bryostatins (macrocyclic lactones), the indole alkaloids such as teleocidin, the polyacetates such as aplysiatoxin, and the iridals. A critical finding is that these ligands do not all induce similar biological responses upon binding. For example, whereas phorbol 12-myristate 13-acetate (PMA) is the paradigmatic mouse skin tumor promoter [3], we have shown that prostratin 13-acetate and 12-deoxyphorbol 13-phenylacetate are anti-tumor promoting [4], as is bryostatin 1 [5]. Reflecting such activities, bryostatin 1 and PEP005 (ingenol 3-angelate) are currently the subject of numerous clinical trials as anti-cancer agents (www.clinicaltrials.gov) and prostratin provides a model for overcoming resistance of cells latently infected with HIV to HAART therapy [6].

Among novel PKC ligands, the bryostatins have proven to be of particular interest [7]. Most all of the focus has been on bryostatin 1, with occasional studies examining other derivatives. Although the bryostatins function *in vitro* as activators of PKC, paradoxically in many cellular systems and for many biological endpoints they fail to induce the responses induced by the typical phorbol esters and, if administered in combination with phorbol ester, block response to the phorbol ester, showing that their failure to induce these responses is not due to instability. Mechanistic comparison reveals numerous differences that could contribute to these opposing outcomes. Bryostatin 1 shows a transient response followed by loss of responsiveness [8]. Bryostatin 1 may cause more rapid down regulation of some PKC isoforms [9,10]. Bryostatin 1 shows a unique pattern of down regulation of PKC delta, with down regulation at low concentrations but protection from down regulation at higher concentrations [11,12]. Finally, bryostatin 1 causes a distinct pattern of membrane translocation of PKC delta. Whereas PMA treatment causes initial translocation to the plasma membrane followed by subsequent distribution between plasma and internal/nuclear membranes, bryostatin 1 causes the initial translocation primarily to the internal membranes [13,14]. A critical conceptual question is whether these multiple differences in biology and in mechanism are linked to the same structural features of bryostatin 1 or whether specific structural features drive different aspects of biological response.

The small number and limited diversity of natural bryostatin derivatives, together with the daunting synthetic challenge of chemical synthesis of the bryostatins, has greatly limited understanding of bryostatin structure-activity relations. The exciting recent advances in the chemical synthesis of bryostatin and bryostatin analogs have now shattered this impasse [15]. In their attempts to identify which features of the bryostatin 1 were dispensable for activity, thereby permitting the design of bioequivalent simplified structures with correspondingly simplified synthetic routes, the Wender group argued that the A- and B-rings of the molecule functioned as a spacer domain, whereas the active pharmacophore resided in the lower half of the molecule [16]. Experimental support for this view was provided through extensive structural comparison, showing that PKC binding activity was retained in such derivatives, and is consistent with computer modeling, indicating that it is the lower portion of the bryostatin structure which inserts into the binding cleft of the C1 domain [17].

A critical issue, however, is which structural elements confer the unique features of bryostatin 1 biological response, rather than simply PKC binding activity, since interest in the bryostatins as therapeutic agents is driven by their distinct activity as compared to the tumor promoting phorbol esters. While PKC binding activity may be necessary for activity, we found that it was not sufficient to confer a bryostatin 1-like pattern of biological response. The bryostatin derivative Merle 23, which differs from bryostatin 1 only in that it lacks four substituents in the so-called "spacer domain", behaved in the U-937 human leukemia cell line like a phorbol ester, not like bryostatin 1 [18] (Fig. 1). Merle 23, like PMA, inhibited cell proliferation and induced attachment, whereas bryostatin 1 failed to induce either response and, in combination with Merle 23 or PMA, antagonized the response to the latter agents.

It is very important to emphasize, however, that the U-937 cell system is only one of the many systems in which the bryostatins induce a distinct pattern of biological response compared to the phorbol esters. As an initial step in developing a more robust understanding of the relationship between structural features of bryostatin analogs and their biology, we have characterized in some detail the responses of Merle 23 with those to bryostatin 1 and PMA in a second system in which bryostatin 1 acts differently from the phorbol esters. In the LNCaP human prostate cell line, phorbol esters inhibit proliferation and induce apoptosis, whereas bryostatin 1 has much less effect. Previous careful characterization of this system by others has highlighted the roles of PKC delta and tumor necrosis factor alpha in these responses, but multiple other PKC isoforms and pathways have been implicated as well [14,19–21]. We report here that, in this system, Merle 23 can be bryostatin-like, phorbol ester-like, intermediate in activity between the two, or be more active than either, depending on which specific biological or mechanistic endpoint we characterize in this system. A crucial conclusion from our findings is that the distinction between the actions of bryostatin 1 and phorbol ester is not all-or-none but rather can be dissected through structural modification. Bryostatin analogs thus should provide a powerful

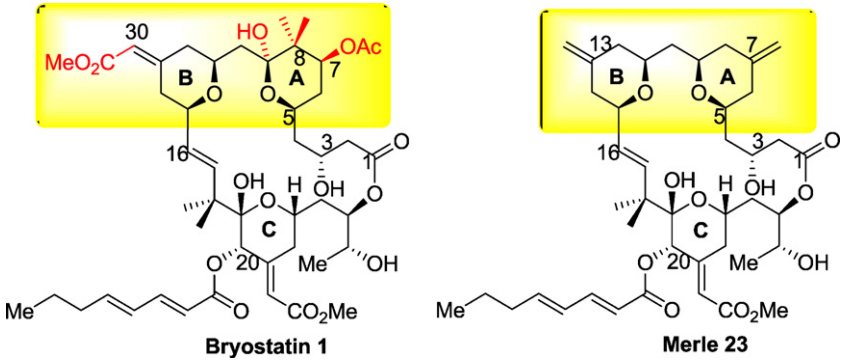

Fig. 1. Comparison of the structures of bryostatin 1 and Merle 23. The region of difference between bryostatin 1 and Merle 23 is highlighted in yellow and the specific substituents of bryostatin 1 which are lacking in Merle 23 are shown in red.