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Current Status and Future Prospectives of C1 Domain Ligands as Drug Candidates

Running title: C1 domain ligands in drug development

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ABSTRACT

Protein kinase C (PKC) comprises a family of ten isoforms that play roles in diverse cellular processes such as proliferation, apoptosis and differentiation. PKC isoforms respond to G-protein coupled receptor- and receptor tyrosine kinase-signaling via binding the second messenger diacylglycerol with C1 domains. Aberrant signaling through PKC isoforms and other C1 domain-containing proteins has been implicated in several pathological disorders. Drug discovery concerning C1 domains has exploited natural products and rationally designed compounds. Currently, molecules from several classes of C1 domain-binding compounds are in clinical trials; however, still more have the potential to enter the drug development pipeline. This review gives a summary of the recent developments in C1 domain-binding compounds.

KEY WORDS

Protein kinase C; C1 domain; drug discovery; cancer; Alzheimer's disease; bryostatins; ingenol-3-angelate; DAG-lactones

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INTRODUCTION

Protein kinase C (PKC) is a family of at least ten isoenzymes involved in the regulation of cell morphology and various cell functions such as proliferation, metabolism and apoptosis. PKCs mediate signals that originate from the activation of G-protein-coupled receptors and receptor tyrosine kinases and lead to phospholipase C activation and the subsequent hydrolysis of cell membrane phospholipids [1]. PKC isoforms are classified into three subgroups according to their structure and activation mechanisms. Classical/conventional PKCs (cPKCs: α, βI, βII and γ) require calcium and diacylglycerol (DAG) for activation and novel PKCs (nPKCs: δ, ε, η and θ) are activated by DAG. Atypical PKCs (aPKCs: ζ and ι/λ) require neither calcium nor DAG (Figure 1) [2, 3]. The activation of PKC is controlled by the regulatory region in the amino terminal part of the enzyme, which, in the case of classical and novel isoforms, contains the C1 domain. The C1 domain of cPKCs and nPKCs is a duplicated cysteine-rich sequence (containing C1a and C1b subdomains) that binds endogenous DAG and tumor-promoting phorbol esters. Binding of either of these ligands leads to the translocation of the enzyme to cell membranes and a subsequent conformational change unveiling the catalytic site and leading to enzyme activation. The C1 domain of atypical PKCs is unresponsive to DAG and phorbol esters.

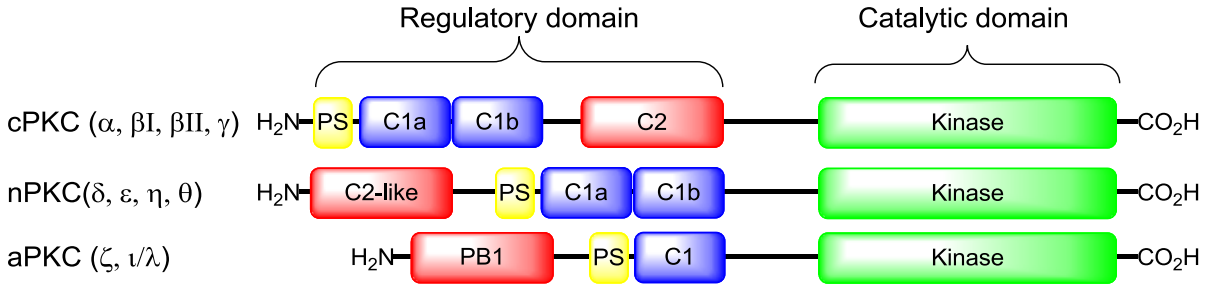

Figure 1. Structures of protein kinase C isoforms. PS = pseudosubstrate, PB1 = phox-Bem1.

There is a large body of evidence supporting the potential of PKC isoforms as therapeutic targets in cancer [4, 5], immunological diseases [6, 7], cardiovascular diseases [8-10] and neurological diseases [11], especially Alzheimer's disease (AD) [12-14]. However, proper target validation is possible only when potent and specific ligands are available. The conventional route of producing kinase inhibitors by targeting the ATP binding site has not been successful with PKC because most of the inhibitors reported in the literature are poorly selective. Some degree of selectivity has been achieved with enzastaurin (LY317615) and ruboxistaurin (LY333531), which have been reported to be selective for PKCβ at nanomolar concentrations, although they also inhibit other kinases [15, 16]. These compounds have been studied in phase III clinical trials for the treatment of cancer and diabetic complications, respectively, but their efficacy has not been proven yet. Problems with achieving selectivity are not surprising because there are potentially more than 500 kinases [17] with relatively homologous ATP-binding sites. Moreover, the 3D crystal structures of only three PKC catalytic domains (β, θ and ι) have been resolved. Therefore, targeting the regulatory part of PKC could provide a way to develop PKC selective compounds.

The C1 domain was first identified in PKC, but later six other protein families with a DAG and phorbol ester-responsive C1 domain were identified: the protein kinase D (PKD), the chimaerins, the guanyl nucleotide-releasing proteins RasGRPs, the Unc-13 scaffolding proteins, the myotonic dystrophy kinase-related Cdc42-binding kinases (MRCKs), and the DAG kinase (DGK) isoforms β and γ. PKD, DGK and the chimaerins are involved in the regulation of cell growth, proliferation and motility, among other things, and may therefore be implicated in the pathology of cancer [18-20]. RasGRPs and DGK regulate immune cell signaling [20, 21], whereas Munc13 isoforms (the mammalian homologs of the yeast Unc-13) are involved in exocytotic processes [22]. MRCK is known to control cytoskeletal

reorganization [23] – a process that is also regulated by PKCs, DGKs and the chimaerins [24]. Thus, PKC is not the only potential drug target among C1 domain-containing proteins.

At present, the disease models (both *in vitro* and *in vivo*) in which the effects of C1 domain ligands have been characterized are mainly based on the proposed role of PKC in the pathophysiology of cancer and AD. In cancer, the differential effects of PKC isoforms on cell proliferation and apoptosis form the basis for targeting PKC. In the central nervous system, PKC regulates processes linked to memory and learning [12]. In AD, certain PKC isoforms promote the processing of amyloid precursor protein (APP) via the non-pathological pathway, thereby causing a relative decrease in pathological Aβ levels [12]. In addition, neuroprotection in stroke [10], cardiac failure and cardioprotection during ischemia [9] as well as diabetic complications [15], autoimmune diseases [7] and HIV-1 infection [25] are relevant in considering possible indications for PKC or C1 domain-targeted treatment.

The use of the C1 domain as a drug target has aroused interest in several research groups [26]. Compared to the ATP binding site, the C1 domain possesses several advantages as a drug target. First, the number of C1 domain-containing proteins is relatively small. Second, targeting the C1 domain provides the possibility of developing PKC activators. Third, the PKC regulatory domain may have biological functions independent of catalytic activity [27-29], and the C1 domain can act as a protein-interaction module [30]. Fourth, targeting the C1 domain provides a way to target other C1 domain-containing proteins in addition to PKC, thereby enhancing the effect on the signaling pathways that those proteins regulate at different levels. Several compounds targeted to the C1 domain have entered clinical trials, providing further validation for the C1 domain as a drug target.

Most of the C1 domain ligands currently known are either derived straight from nature or are derivatives or analogues of naturally occurring compounds. The chemistry of the naturally derived C1 domain ligands (including phorbol esters, bryostatins, teleocidins and

aplysiatoxins) is complex and their commercial production and chemical modification is thus generally unfeasible. Therefore, two main strategies have been applied to develop novel C1 domain ligands by (1) simplifying the chemical structure of the natural ligands (bryostatin analogues, benzolactams) or by (2) using DAG as starting point and working towards more constrained and complex structures (DAG-lactones). Additionally, C1 domain ligands have been discovered by screening campaigns and structure-based design; 5-(Hydroxymethyl)isophthalates, *N*-benzyladriamycin-14-valerate (AD 198), calphostin C and resveratrol are examples of these ligands. This review focuses on recent advances in the development of C1 domain-targeted compounds and evaluates their potential in drug development.

THE CRYSTAL STRUCTURE OF THE PKCδ C1B DOMAIN

Rational drug design for targeting the C1 domain containing proteins has been hampered by the lack of solved crystal structures. Consequently, pharmacophores obtained from natural products have been used in the development of analogues targeted to the C1 domain. To date, the crystal structure of the C1b domain of PKCδ complexed with phorbol 13-*O*-acetate (**1**, crystallized at 2.2 Å resolution; PDB ID: 1PTR, Figure **2**) is the only available 3D structure of a PKC C1 domain [31]. The crystal structure reveals a domain whose "top" third is hydrophobic and that contains a deep, narrow and polar groove that binds diacylglycerol (DAG, **2**) and phorbol esters (**3**). The "central" part of the domain is hydrophilic and the "bottom" third of the domain contains negatively charged amino acids. The C1 domain bound to DAG or phorbol ester is thought to be partly buried in the membrane bilayer [32] because the ligands can act as caps on the top part of the domain to produce a continuous hydrophobic surface. To further stabilize the penetration of the C1 domain into the bilayer, the positively

charged amino acids in the central part of the domain could interact with anionic membrane phospholipids and the negatively charged amino acids in the bottom of the domain could face the cytoplasm.

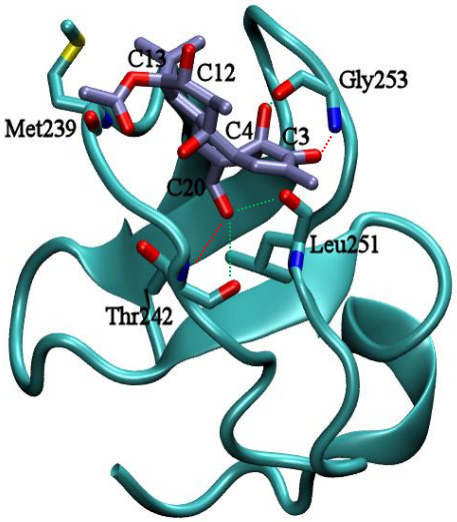

Figure 2. The crystal structure of the PKCδ C1b domain complexed with phorbol 13-*O*-acetate (**1**). The pivotal polar functional groups of the phorbol ester involved in C1 domain recognition include the hydroxyl groups in the carbon atoms C4 and C20 and the carbonyl group on C3 [31]. The carbonyls of Gly253, Leu251 and Thr242 are hydrogen-bond acceptors (green dotted lines), and the amide protons of Gly253 and Thr242 are hydrogen-bond donors (red dotted lines). The figure was created using VMD (v.1.8.6., University of Illinois, USA).

The binding site of DAG/ phorbol esters in the regulatory domain forms a deep polar groove between two β-sheets (Figure **2**). The carbonyls of Leu251 and Thr242 at the bottom of the groove act as hydrogen-bond acceptors for the hydroxyl of the C20 of phorbol esters. In addition, the amide hydrogen of Thr242 can act as a hydrogen-bond donor for the hydroxyl of the C20 of phorbol esters. Furthermore, the carbonyl and the amide proton of Gly253 interact with the C4 hydroxyl and the C3 carbonyl of phorbol 13-*O*-acetate, respectively. The same interactions to Leu251 and Thr242 can be found with the C3 hydroxyl (Figure **3**). In addition, the carbonyl group of the acyl moiety of DAG at C1 has the same interaction with the protein

as the C3 carbonyl of phorbol esters. In phorbol esters, the C13 carbonyl group (e.g., compound **4**) is essential for high-affinity binding while the C12 carbonyl group is not (e.g., compound **5**) [33-35]. Although the crystal structure does not reveal any interactions of the C13 carbonyl to the protein, it is speculated that this carbonyl is bridged *via* a water molecule to the backbone carbonyl of Met239, to the bilayer and/ or to the C9 hydroxyl [36, 37]. Furthermore, no interaction between the C9 hydroxyl and the protein can be observed in the crystal structure, but it has been shown that the C9 hydroxyl is involved in interactions with the bilayer. To accomplish PKC activity, the absolute configuration of the hydroxyl-bearing carbon atom C4 in phorbol esters has to be *R*, the respective *S*-configuration (e.g., compound **6**) provides an inactive compound [38]. However, the C4 hydroxyl is not essential to provide high binding affinity to PKC [39].

In addition to the crystal structure of the PKCδ C1b domain, Canagarajah *et al*. reported the crystal structure of the uncomplexed and intact β2-chimaerin at 3.2 Å resolution (PDB ID: 1XA6) [40]. The C1 domain of β2-chimaerin was closely superimposable on the C1b domain of PKCδ. However, in the crystal structure of β2-chimaerin, a part of the N-terminus (residues 27-34) forms a helix that covers the binding groove of the C1 domain. In addition, Gln32 forms hydrogen bonds with Gly235 in the "top" of the binding groove (Gly235 corresponds to Gly253 in the PKCδ C1b domain, Figure **2**).

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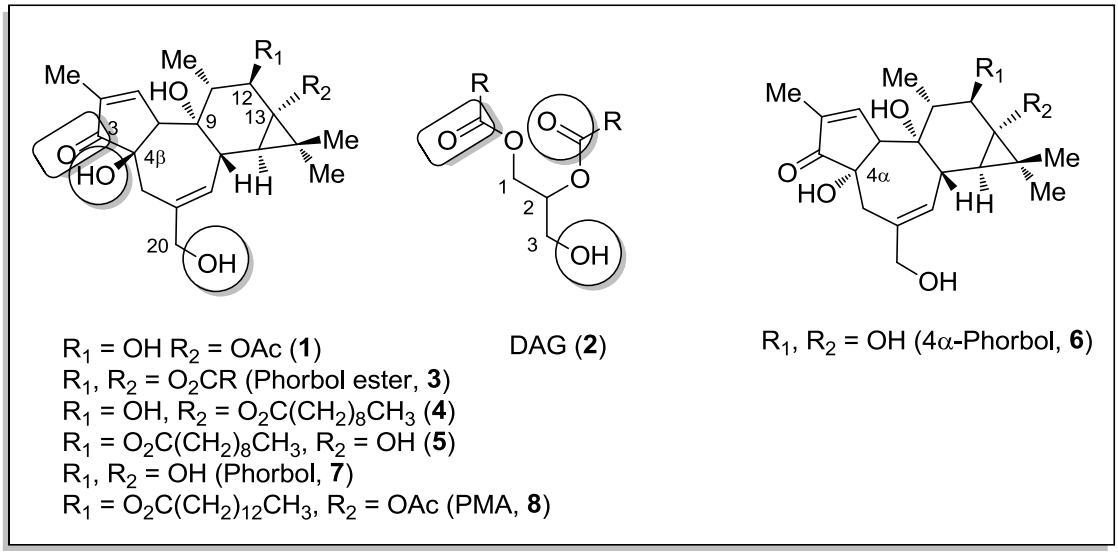

Figure 3. Structures of phorbols (**1** and **3-8**) and DAG (**2**). Functional groups that interact with the C1 domain are highlighted.

BRYOSTATIN 1 AND ITS SYNTHETIC ANALOGUES

The highly oxygenated macrocyclic lactones bryostatins were originally isolated from the marine bryozoan *Bugula neritina* [41]; however, the symbiont bacteria might be the actual sources of the compounds [42]. There are at least 20 naturally occurring bryostatins, with bryostatin 1 (**9**, Figure **4**) being the prototype that has been extensively examined. Bryostatin 1 shows very high affinity towards PKC and exhibits unique biological effects when compared to phorbol esters. In general, bryostatin 1 has been reported to act as an antineoplastic agent, to induce differentiation in hematopoietic cells, to activate effector cells of the immune system, to reverse neuronal damage caused by stroke and to rescue long-term memory after ischemia. It has proceeded to clinical trials as a potential treatment for different cancers and AD. In addition, several synthetic analogues of bryostatin 1 have been prepared, and structure-activity studies have been performed.

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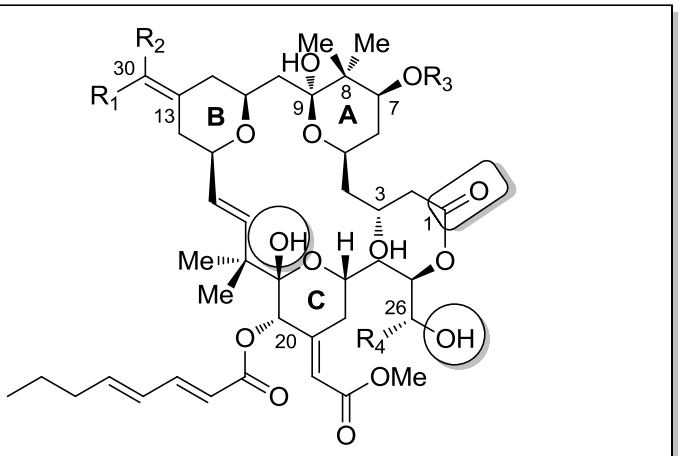

Compound	R_1	R_2	R_3	R_4	K_i (nM)	Ref.
Bryostatin 1 (**9**)	CO_2Me	H	Ac	Me	1.35	[43]
Bryostatin 2 (**10**)	CO_2Me	H	H	Me	5.9	[43]
11	H	H	Ac	Me	0.52	[52]

Figure 4. Structures of bryostatins (**9-11**). Functional groups that interact with the C1 domain are highlighted.

Structure-activity studies

Bryostatin 1 binds to PKCα with a K_i of 1.35 nM [43]. The bryostatins are highly complex macrolactones containing several stereocenters, and their total syntheses require over 40 linear steps [44-46]. Therefore, the Wender group developed a synthesis route for simplified derivatives of bryostatin 1 with a significantly reduced number of reaction steps [47, 48]. Removal of the methyl substituent at C26 of compound **13** (K_i = 3.4 nM of PKC) [49] gave compound **12**, which showed higher affinity (K_i = 0.25 nM of PKC) [43], as predicted by the pharmacophore of phorbol esters (**3**) (Table **1**). Interestingly, the addition of an acetoxy substituent to C7, similarly to bryostatin 1, reduced the K_i value to 13 nM (compound **14**), and the addition of a hydroxyl group to C7 (analogues **15** and **16**) reduced the binding affinity even further by three to four orders of magnitude. On the other hand, bryostatin 2 (**10**), with a hydroxyl group at C7, shows only a slight reduction in binding affinity compared to bryostatin 1 [43]. It has been speculated that these differences in binding affinities are due to the C8 *gem*-dimethyl groups, which are able to shield a polar C7 substituent. This hypothesis

was proven correct by the corresponding pivaloyloxy (analogue **17**) and methylene (analogue **18**) derivatives, which showed better binding affinities (K_i of 6.6 and 5.3 nM, respectively) compared to compound **14**. Furthermore, the Blumberg group showed that the removal of the methyl and hydroxyl groups in ring A of bryostatin 1 gave compounds that retained excellent binding to PKCα (0.7-3.0 nM, compounds **21-24**) but that evoked phorbol-like tumor promoting responses [50, 51]. Alternatively, compound **11**, which has the same C7-C9 substituents as bryostatin 1 and lacks only the C30 methoxy carbonyl group, showed a K_i value of 0.52 nM for PKCα but is also a functional antagonist of PMA (**8**) [52]. In contrast to these findings, derivatives **19-20** and **25-27** synthesized by the Wender group, lacking all the A-ring substituents but having the C13 substituted, showed high affinities to PKC (K_i = 0.67-2.5 nM), and compounds **25-27** inhibited the growth of K562 and MV411 leukemia cells [53, 54]. Furthermore, different esters at C20 do not seem to affect binding [52, 55], and the C3 hydroxyl group is pivotal for high binding affinity [49]. Taken together, the SAR studies suggest that the C26 methyl of bryostatin 1 is not essential for its PKC binding activity and that the A- and B-rings can be modified to achieve anti-tumor promoting analogues of bryostatin.

Table 1. Structures and binding affinities of bryostatin derivatives (**12-27**). Functional groups that interact with the C1 domain are highlighted.

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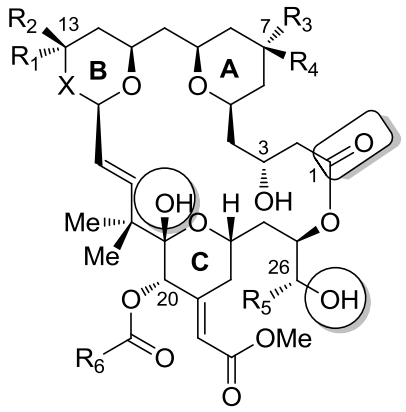

Cmpd	X	R_1	R_2	R_3	R_4	R_5	R_6	K_i (nM)	Ref.
12	O	H	H	H	H	H	*n*-C_7H_{15}	0.25 [a]	[48]
13	O	H	H	H	H	Me	*n*-C_7H_{15}	3,4	[49]
14	O	H	H	H	OAc	H	*n*-C_7H_{15}	13	[43]
15	O	H	H	H	OH	H	*n*-C_7H_{15}	100	[43]
16	O	H	H	OH	H	H	*n*-C_7H_{15}	1000	[43]
17	O	H	H	H	O_2CBu-*t*	H	*n*-C_7H_{15}	6,6	[43]
18	O	H	H	-	$=CH_2$	H	*n*-C_7H_{15}	5,3	[43]
19	O	H	H_2C=CH$CH_2O_2CCH_2$	H	H	H	*n*-C_7H_{15}	1,2	[54]
20	O	H	H_2C=$CHCH_2$	H	H	H	*n*-C_7H_{15}	0,67	[54]
21	CH_2	-	H_2C=	-	$=CH_2$	Me	Ph	0,70	[50]
22	CH_2	-	H_2C=	-	$=CH_2$	Me	*n*-C_7H_{15}	1,05	[50]
23	CH_2	-	H_2C=	H	OAc	Me	*n*-C_7H_{15}	3,0	[51]
24	CH_2	-	H_2C=	-	$=CH_2$	Me	C_7H_{11} [b]	0,70	[50]
25	CH_2	-	(*Z*) MeO_2CCH=	H	H	H	*n*-C_7H_{15}	0,9	[53]
26	CH_2	-	(*E*) MeO_2CCH=	H	H	H	*n*-C_7H_{15}	2,5	[53]
27	CH_2	-	H_2C=	H	H	H	*n*-C_7H_{15}	1,63	[53]

[a] Wender *et al.* also reported a K_i-value of 3.1 nM for the same derivative [53]. [b] C_7H_{11} = (1*E*,3*E*)-hepta-1,3-dienyl.

Interaction with PKC and other C1-containing proteins

Bryostatin 1 binds to PKC with high affinity but without showing considerable isoform specificity in binding or activation [56]. It competes for binding with DAG and phorbol esters, suggesting that they share the same binding site; however, some of the biological effects caused by these agents differ. In general, PMA is a tumor promoter, while bryostatin 1

has shown antiproliferative effects. Furthermore, bryostatin 1 acts as a partial functional antagonist, often suppressing or reversing the effects induced by PMA [57-59]. The mechanism behind the functional differences has been an area of intense investigation, but no single mechanism has been revealed. Antagonism, at least for leukemia cell differentiation, is non-competitive [57].

At the cellular level, bryostatin 1 shows differential effects on PKC isoform translocation and down-regulation compared to PMA, which might explain some of the functional differences [60-64]. Bryostatin 1 depends on both the C1a and C1b domains for the induction of PKC translocation, whereas PMA and other tumor promoters mainly depend on the C1b domain, a pattern that was proposed to correspond with tumor-promoting activity [65]. Additionally, the bryostatin 1-induced translocation is slower and the localization of the PKC isoforms is different compared to PMA. Bryostatin 1 has been shown to induce more rapid down-regulation of PKCα and PKCβ compared to PMA [66, 67]. Furthermore, PKCδ is resistant to bryostatin 1-induced down-regulation at high concentrations, but not at low concentrations [60]. The C1a domain and the catalytic domain might be involved in protection from down-regulation [61, 62]. Because PMA does not protect PKC from down-regulation, unique structural aspects of bryostatin 1 are likely to be involved.

Specific to bryostatin 1 are its biphasic concentration-dependent effects in many biological responses, such as enhancing the proliferation and cytokine secretion of human mononuclear cells [68], inducing c-Jun protein in NIH 3T3 cells [60], sensitizing HeLa cells to cisplatin-induced cell death [69], inducting the proliferation of HOP-02 cells [63], and activating PKD [70]. The underlying mechanism(s) is not clear but could involve differential activation of distinct PKC isoforms [63, 69]. Furthermore, many of the cellular effects caused by bryostatin 1 are thought to depend on binding to PKC isoenzymes; however, activations of

other C1 domain containing proteins such as PKD [70, 71], RasGRPs [64, 72, 73], and β-chimaerin [74] have been reported.

Antineoplastic effects

Bryostatin 1 and several of its synthetic analogues have shown antineoplastic effects or have induced differentiation in several cancer cell lines [43, 48, 50-53, 75, 76], in primary cancer cells [77], and in *in vivo* tumors [78]. However, stimulation of proliferation or antiapoptotic effects has also been reported [64, 79]. Some of the antineoplastic effects of bryostatin 1 or its analogues occur via PKC isoforms, but some are PKC-independent [64, 71, 80, 81]. Bryostatin 1 has been shown to circumvent resistance to drug-induced apoptosis and to potentiate the effects of other oncolytic agents such as imatinib [82], vincristine [83], and paclitaxel [84].

Immunomodulatory effects

Bryostatin 1 is a potent activator and modulator of host immune effector cells, which may indirectly cause tumor cell death. Bryostatin 1 can enhance the proliferation and activation of human T and B cells [85, 86], induce the expression of cytokine receptors [86], enhance the release of cytokines [85, 87], and enhance the immunogenicity of chronic lymphocytic leukemia (CLL) cells [88].

Clinical trials

Because of the remarkable antineoplastic effects observed in *in vitro* studies and in preclinical animal experiments, the safety and efficacy of bryostatin 1 have been examined in several clinical trials for the treatment of different cancers either as a single agent or in combination with other established anticancer agents. However, as a single agent, bryostatin

1 has not shown efficacy in phase I and II trials for treatment of cancers such as melanoma, renal, colorectal, non-Hodgkin lymphoma, soft tissue sarcoma and head and neck cancers (reviewed in [89, 90]). Therefore, there are clinical trials focused on using bryostatin 1 in combination with other established antitumor drugs. Most of the combination studies have, however, been disappointing, and bryostatin 1 has shown moderate efficacy in only a few trials (Table **2**, [91-98]). In addition, myalgia is a major adverse effect that has limited the use of bryostatin 1 at doses higher than 40-50 μg/m^2. Despite the disappointing results of bryostatin 1, researchers are hoping to obtain better results and reduced toxicity with new bryostatin analogues [92].

Table 2. Clinical trials of bryostatin 1 in combination with antitumor agents.

Bryostatin with	**Phase**	**Cancer**	**Result**	**Ref.**
Cisplatin	I	Non-hematologic tumors	PR in 4 out of 53 patiens [a]	[91]
Gemcitabine	I	Non-hematologic malignancies	PR in 2 out of 36 patients, 8 had stable disease	[96]
Fludarabine	I	CLL and indolent lymphoma [b]	CR in 4 out of 59 patients, 20 had partial remission [c]	[95]
Interleukin-2	II	Advanced kidney cancer	PR in 1 out of 33 patients, 1 stable disease	[97]
Paclitaxel	II	Gastric and gastroesophageal cancer	PR in 10 out of 35 patients	[98]
		Advanced esophageal cancer	PR in 6 out of 22 patients	[94]
		Advanced pancreatic cancer	Inactive	[93]
Vincristine	II	Non-Hodgkin lymphoma	CR in 2 and PR in 2 out of 14 patiens	[92]

[a] PR = partial response; [b] CLL = chronic lymphocytic leukemia; [c] CR = complete response.

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Effects on central nervous system

A new field of investigations of bryostatin 1 and its analogues concerns their effects on the central nervous system. Bryostatin 1 and its analogue **12** [48] have been shown to enhance the secretion of nontoxic soluble APPα (sAPPα) in fibroblasts from AD patients [99], and bryostatin 1 has been shown to reduce the toxic $A\beta_{40}$ and $A\beta_{42}$ peptides in the brains of transgenic mice and to improve behavioral outcomes in an open field test [100]. Due to these very promising results, bryostatin 1 is now about to enter clinical trials to test its efficacy in AD patients [101].

PKC is also implicated in stabilizing the mRNAs of proteins that might be important in the processes of memory and learning [102]. The stabilization is evoked via RNA-binding proteins (RBPs), and some of the most important RBPs are neuronal Embryonic Lethal Abnormal Vision (nELAV)-proteins. These proteins bind to the adenine and uridine rich-motifs of the 3'UTR-region of their target mRNA and can affect mRNA splicing, nuclear transport, stability and translation [102]. The nELAV proteins have been shown to be up-regulated by bryostatin 1 in human SH-SY5Y neuroblastoma cells and in rat brain [103]. Through the phosphorylation and up-regulation of nELAV proteins, PKC could positively influence the expression of genes important in neurogenesis, memory formation and, possibly, neuroregeneration [102].

Global cerebral ischemia selectively injures the pyramidal neurons in the dorsal hippocampal CA1 area, causing dramatic and long-lasting impairment of spatial learning and memory [104, 105]. In an animal model of experimental global ischemia, chronic bryostatin 1 administered for 5 weeks, with the first dose given 24 hours after the induced injury, restored the performance of the rats in memory and learning tasks [104]. Bryostatin 1 enhances neurotrophic activity and neuronal survival, prevents synaptic loss and induces

Boije af Gennäs G et al. Curr Top Med Chem. 2011; 11(11): 1370-92
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synaptogenesis after ischemia. These effects are remarkable because current thrombolytic therapy cannot repair the damaged brain tissue and must be administered within 3 hours of the stroke. The action of bryostatin 1 after ischemia is also long lasting [105]. Rats tested 4 months after the administration of the last bryostatin 1 dose performed as well as the control group in tests of spatial learning and memory. Furthermore, bryostatin 1 rescues even previously learned experience.

INGENOL DERIVATIVES

The sap of the plant *Euphorbia peplus* has been used as a traditional remedy to treat skin-related diseases such as warts, corns and skin cancers as well as asthma and migraine [106, 107]. Three major families of macrocyclic diterpenes extracted from *E. peplus*, the ingenane, the pepluane and the jatrophane families [108] have been identified as the source of agents having cytotoxic, proinflammatory and cell-differentiating activity. Ingenol-3-angelate (PEP005, ingenol mebutate, **28**) from the ingenane family has been extensively studied and is considered a potential agent for the treatment of skin tumors [107].

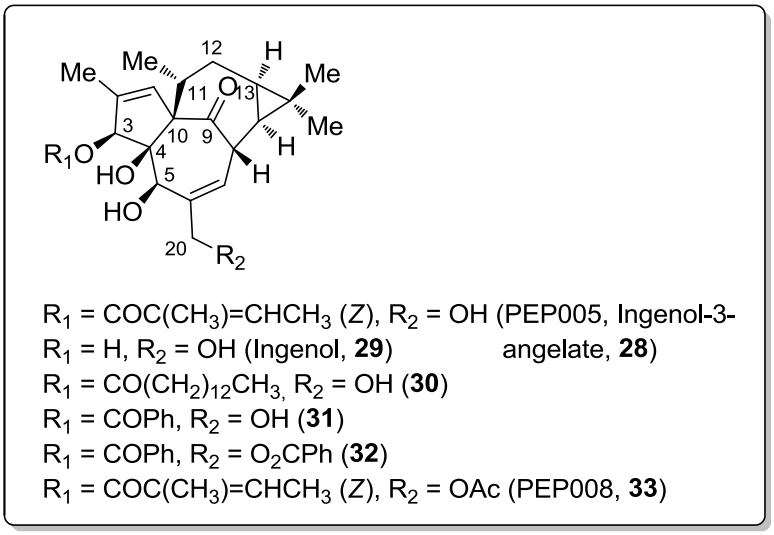

Figure 5. Structures of ingenols (**28-33**).

Boije af Gennäs G et al. Curr Top Med Chem. 2011; 11(11): 1370-92
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Structure-activity studies

Even though they are structurally related to phorbol (**7**), ingenols (**28-33**) contain some considerable differences within their structures (Figure **5**). When compared with the seven-membered ring of the phorbols, the ingenols have an additional hydroxyl group at C5, and the C9 hydroxyl of the phorbols is in an oxidation state of a carbonyl. Additionally, the six-membered ring of the phorbols corresponds to the new seven-membered ring in the ingenol structure. Furthermore, the ingenols have no ester groups at C12 or C13, and the C3 carbonyl found in phorbols is replaced with a hydroxyl group in ingenols. These differences in the structures of the corresponding ingenols make them more hydrophilic than the phorbol esters; for example, the clog *P* values of ingenol-3-angelate and PMA (**8**) are 3.89 and 6.65, respectively [109]. The corresponding tetradecanoate (**30**) [110] and benzoate (**31**) [111] derivatives have also been studied.

Interaction with PKC

Ingenol-3-angelate binds to PKC with subnanomolar affinity that shows no selectivity between different PKC isoforms. However, it activates PKCδ more potently than PKCα *in vitro* [109]. When compared to PMA, ingenol-3-angelate is less potent in stabilizing the interaction of the PKCδ C1b domain with lipid membranes. The difference in the membrane interaction is thought to correlate with the partial agonism of ingenol-3-angelate in inducing PKCα activation and with the partial antagonism of PKC activation by PMA and 1,2-dioctanoyl-*sn*-glycerol [109]. Ingenol-3-angelate induces a distinct pattern of translocation of PKCδ when compared to PMA, with ingenol-3-angelate inducing translocation initially to the nuclear and perinuclear membrane, and PMA inducing translocation to the plasma membrane

[109, 112, 113]. The differences in membrane interaction and translocation probably mirror the different lipophilicity between the two agents [109].

Effects on cell proliferation

In general, ingenol derivatives are considered antineoplastic agents, although co-carcinogenic or anti-apoptotic activity has also been reported [114-117]. Ingenol-3-angelate has been shown to inhibit the proliferation of different cancer cell lines [109, 116, 118-120] as well as primary acute myeloid leukemia (AML) cells [112, 121]. In contrast, normal primary human neonatal fibroblasts [122] and normal CD34+ cord blood myeloblasts were resistant to permanent growth arrest and apoptosis, respectively [112].

In myeloid leukemia cell lines and in primary AML blasts, ingenol-3-angelate induced apoptosis at low nanomolar concentrations (< 20 nM), apparently via the activation of PKCδ [112]. Normal CD34+ myeloblasts did not undergo apoptosis but were induced to differentiate with higher concentrations of ingenol-3-angelate (20 nM-2 μM). The compound also induced cell differentiation in some leukemia cell lines, as assessed by the increased expression of CD11b protein [112], as well as in primary AML cells, where the expression of leukocyte markers CD11b and CD14 increased and the expression of stem cell marker CD117 decreased [123]. Of the other ingenol ester derivatives, ingenol-3,20-dibenzoate (**32**) and its analogues induced apoptosis in Jurkat cells via a PKC-independent pathway [114]. The PKC-activating pathway and the apoptosis pathway seemed to be separate, and only those derivatives bearing a benzoate at C20 could induce apoptosis. In addition, a free hydroxyl at C5 was critical for PKC activation.

Permanent growth arrest, called senescence, was reported as the mechanism of cytostatic effect induced by low doses (0.1-1 μg/ml) of ingenol-3-angelate in certain melanoma cell lines [122] and of 20-*O*-acetylingenol-3-angelate (PEP008, **33**) in solid tumor (melanoma,

breast, colon) cell lines [124]. In both studies, the cells exhibited irreversible, PKC-dependent G_1 and G_2/M arrest, and the senescence process was dependent on the overactivation of the ERK1/2 kinase pathway, but no correlation was detected between the induction of senescence and PKC isoform expression. Activation of the ERK 1/2 pathway has been reported to occur in both sensitive and resistant cells [121, 122, 124]; however, the kinetics of the activation appear to be important for the induction of apoptosis in leukemia cells, with sustained activation (> 6 hours) occurring in the sensitive cells and transient activation (< 6 hours) in the resistant cells [121].

In addition to the activation of the ERK 1/2 pathway, ingenol-3-angelate treatment has been shown to activate other MAPK pathway kinases such as JNK [120] and p38 [119, 120]. Furthermore, inhibition of the phosphatidyl 3-kinase/ Akt signaling pathway by ingenol-3-angelate has been reported in the colon cancer cell line Colo205 [120]. However, the modulation of Akt signaling was apparently PKC-independent. Recently, ingenol-3-angelate was shown to inhibit T-cell apoptosis via the activation of PKCθ [117]. This PKC isoform is expressed in T cells but absent in myeloid leukemia cells. However, the myeloid NB4 leukemia cells were rendered resistant to apoptosis by the ectopic expression of PKCθ.

Immunomodulatory effects

In addition to direct anti-leukemic actions, ingenol-3-angelate shows immunomodulatory effects [123, 125]. Ingenol-3-angelate enhances cytokine release after chemotherapy-induced cytopenia in T cells from AML patients. The release of IL-2 may explain the observed increase in T cell proliferation, whereas TNFα and INFγ may have anti-leukemic effects [125]. Furthermore, ingenol-3-angelate enhances the release of T-cell chemotactic chemokines and cytokines from primary AML cells [123]. Taken together, these results suggest that ingenol-3-angelate could be used as an adjuvant in AML therapy.

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Effects on tumors

When applied topically on C57BL/6 or *Foxn1*^{nu} mice bearing subcutaneous tumors of mouse and human origin, ingenol-3-angelate regressed the tumors with great efficacy [118]. The initial mechanism of high doses (LD_{90} 180-220 µM) of ingenol-3-angelate appears to be primary necrosis in the tumor cells. Ingenol-3-angelate is thought to rapidly disrupt the cell membrane by dissolving into it, followed by PKC-independent mitochondrial swelling and cell death [118]. The initial necrosis is associated with a local inflammatory reaction in the cells surrounding the tumor leading to recruitment of neutrophils, which kill residual tumor cells via an antibody-dependent cellular cytotoxicity mechanism [126]. Both the inflammatory phase and apoptosis are thought to depend on PKC activation.

Ingenol-3-angelate induces the activation of neutrophils via the induction of cytokine (MIP-2, TNFα, IL-1B) release, the activation of endothelial cells vital for neutrophil adhesion and extravasation [113, 126], the production of tumoricidal reactive oxygen species by neutrophils, and the production of anti-cancer antibodies [126]. In addition, ingenol-3-angelate has been shown to regress untreated secondary tumors by inducing the generation of anti-cancer CD8 T cells after injection into primary tumors in mice [127].

Clinical trials

Ingenol-3-angelate is currently being developed as a topical treatment for actinic keratosis [107, 128, 129]. Actinic keratoses are pre-cancerous lesions often caused by sun exposure and can lead to skin cancers such as invasive squamous cell carcinoma (SCC) if left untreated. In phase I-III trials, ingenol mebutate (ingenol-3-angelate) gel (0.005-0.05%) has been applied for 2-3 days on the lesions and followed up to 8-12 weeks. Complete clinical

clearance of 28-71% of the treated lesions on head[1] and non-head[2] locations have been reported [128, 129]. The treatments have been well tolerated with only minor adverse events such as erythema, scabbing/crusting and flaking/scaling/dryness. Other potential uses for ingenol-3-angelate include cutaneous warts and other non-melanoma skin cancers, such as SCC and nodular basal cell carcinoma (BCC), leukemia and bladder cancer [101].

INDO- AND BENZOLACTAMS

The teleocidin (**37)** family of indole alkaloids was first isolated from *Streptomyces mediocidicus* [130]. The compounds were found to act as tumor promoters [131] and were subsequently shown to bind to the same receptors as the phorbol esters [132]. The structurally simplest member of the family is (-)-indolactam V (ILV, **34**), which can be produced in large quantities in *Actinomycetes* and has therefore been used as a starting point for the synthesis of new derivatives [58]. Several groups - the Irie, the Kozikowski, and the Endo groups - have carried out research into these compounds, revealed important structure-activity relationships and developed novel indolactam and benzolactam derivatives with improved selectivity profiles (see, for example, [133-135]).

[1] Poster presented by Spencer J. *et al*. at the 68th Annual Meeting of the American Academy of Dermatology meeting, March 5-9 2010, Miami, FL, USA.
[2] Posters presented by Swanson N. *et al*. and Schmieder G.J. *et al.* at the 68th Annual Meeting of the American Academy of Dermatology meeting, March 5-9 2010, Miami FL, USA.

Boije af Gennäs G et al. Curr Top Med Chem. 2011; 11(11): 1370-92
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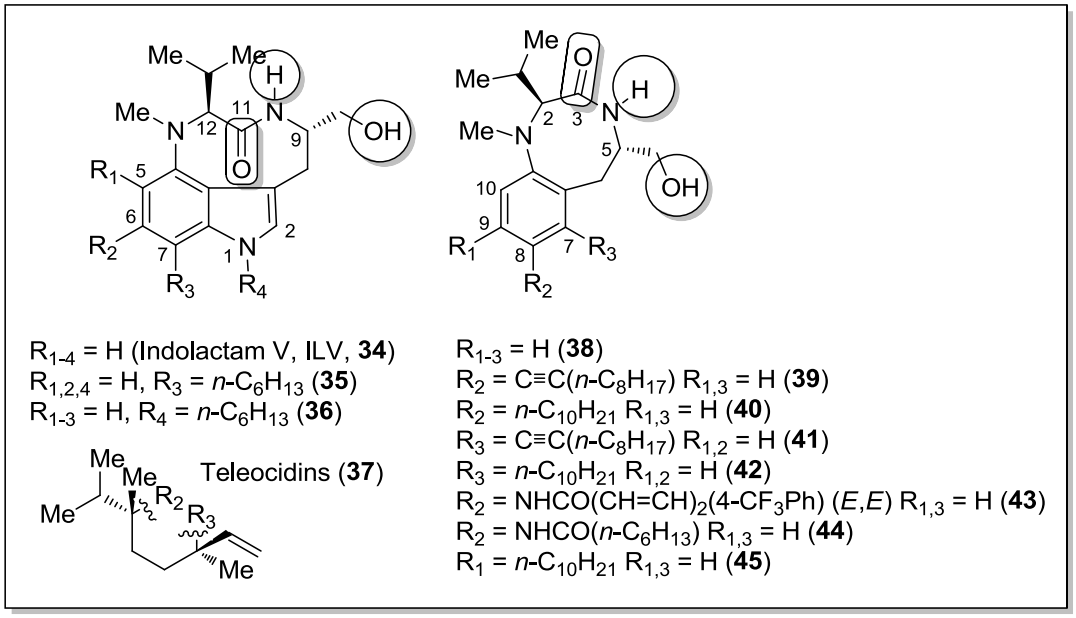

Figure 6. Structures of indo- and benzolactam derivatives (**34-45**). Functional groups that interact with the C1 domain are highlighted.

Structure-activity studies

Similar to the phorbol esters (**3**), the indo- and benzolactams (**34-45**) bind to the C1b domain [136-138]. The primary hydroxyl groups of indolactams and benzolactams form hydrogen bonds to Thr242 and Leu251 in the groove of the C1b domain. In addition, the lactam N-hydrogen is hydrogen-bonded to Leu251. The lactam group was shown to be essential for binding, as the corresponding lactones showed higher K_i values [139]. Furthermore, the C11 and C3 carbonyl groups of indo- and benzolactams, respectively, act as hydrogen-bond acceptors for Gly253. In addition, the lower K_i value of ILV (**34**) compared to compound **38** (80 and 1700 nM, respectively) was explained by CH/π interactions between the indole ring in ILV and Pro241 in PKCδ [135, 140].

The SAR of differently substituted indolactams has been studied intensively, is reviewed in [141] and will not be presented here. The following SAR results have been presented for benzolactams: 1) a hydrophobic substituent in the phenyl ring increases binding to the C1 domain, and position 9 is preferred before positions 8, 10 and 7 (in this order) [142]; 2)

substitution at positions 7 and 10 reduce binding affinity [143-145]; 3) the isopropyl at C2 can be substituted with a long alkyl chain [146]; 4) the (2*S*,5*S*) stereoisomer of the four possible stereoisomers is the most active [147]; 5) a linear alkyl chain with 12-14 carbons or a secondary or cyclic substituent with 11-15 carbons at C2 enhances binding affinity [148]; and 6) expanding or contracting the lactam ring size reduces binding affinity significantly [147, 149, 150].

Kozikowski and co-workers synthesized bivalent indo- and benzolactams to study intramolecular or intermolecular binding between two C1 domains of PKC [151]. Benzolactam **44** (K_i = 225 nM for PKCα) was used as a template and was therefore coupled at C8 with an amide linker to give dimers of the derivative. A clear dependence on the linker length vs. binding affinity was obtained, and the best dimers showed K_i values between 1.8 and 38 nM for PKCα. These bivalent benzolactams showed a modest selectivity for PKCδ (3.2-6.6 times higher affinity for PKCδ, compared to PKCα); however, intramolecular binding between the C1 domains could not be obtained.

Selectivity for PKC isoenzymes

According to the SAR studies, some derivatives are selective for certain PKC isoenzymes. For example, the benzolactams **39** and **40** bind to cPKC isoforms α and β with a certain degree of selectivity as compared to nPKCs δ and ε [152]. With the C7-substituted compound **42**, the opposite is true: it shows an 8-fold selectivity for the nPKC isoform ε over the cPKC isoform α [144]. Furthermore, a series of compounds that show selectivity for C1b domains of nPKC over cPKCs has been developed. Among indolactams studied, compound **35** showed some selectivity for the C1b domain of nPKCs and K_i values of 0.18-0.59 nM [133]. However, when the proton of the nitrogen in the indole ring is substituted with an *n*-hexyl group (compound **36**), the selectivity for the C1b domains of nPKCs increases but with some

loss of binding affinity (K_i = 12-29 nM). [138, 153] Among the benzolactams, the same pattern can be obtained; substitution at C8 (compound **40**) gives higher selectivity for the C1b domains of nPKC than substitution at C9 (compound **45**) [154]. Again, selectivity was increased at the cost of binding affinity.

In addition to PKC C1 domains, indo- and benzolactam derivatives have been shown to bind to the C1 domain of RasGRP with similar affinity as to the PKCδ C1b domain [136].

Effects in experimental models of cancer and AD

Benzolactam derivatives have been studied for their antiproliferative actions on cancer cells and their neuroprotective effects in cell-based models of AD. ILV and several benzolactam derivatives have been reported to inhibit the proliferation of HL-60 leukemia cells [135, 147, 148]. The benzolactam **39** inhibits the proliferation of two breast carcinoma cell lines [152]. The same compound also activates PKCα and restores potassium channel activity and tetraethylammonium chloride (TEA)-induced calcium responses in fibroblasts from AD patients [155]. Furthermore, it enhances sAPP secretion in fibroblasts and PC12 cells [156]. Another benzolactam (**43**, K_i = 11.9 nM for PKCα [137]) increased α-secretase activity and sAPP secretion with a concomitant decrease in β-secretase activity and Aβ40 release in PC12 and SH-SY5Y neuroblastoma cells transfected with human wild-type APP^{695} [157]. These promising *in vitro* results were largely confirmed in *in vivo* experiments with AD transgenic APP[V717I] mice in which the benzolactam **38** enhanced the secretion of sAPP and decreased the secretion of Aβ40 if treatment was initiated early in life [100]. However, more *in vivo* studies are needed to confirm the potential of these compounds as drug candidates.

Induction of differentiation

Although a recognized tumor-promoter, ILV has recently been used as an inducer of human embryonic stem cell (hESC) differentiation [158]. The Melton group used high-content screening to identify small molecules that increase the number of cells expressing the pancreatic transcription factor Pdx1 (Pancreatic and duodenal homeobox 1). ILV directed hESC differentiation into the pancreatic lineage with an EC_{50} of 142 nM, and the effect was totally blocked with PKC antagonists. Even though the PKC isoform responsible for the effect has not yet been identified, the results suggest a role for PKC in pancreatic development and the potential of other PKC or C1 domain-targeted compounds to induce differentiation. Indeed, the authors reported that PMA and benzolactam **43** possess differentiation-inducing properties similar to those of ILV [158].

DAG-LACTONES

Some of the examples of rationally designed C1 domain-binding ligands are the DAG-lactones developed by the Blumberg and Marquez groups. Their initial plan was to design conformationally constrained γ-lactones to overcome the entropial penalty caused by the flexible structure of endogenous DAG (**2**) upon binding to PKC (Figures **3** and **7**) [26, 159]. The potency of these DAG analogues could then be increased by the addition of lipophilic side chains to improve the interaction with the conserved hydrophobic amino acids in the space between the two β-sheets of the C1 domain. Synthesis of the optically pure (2*R*)-DAG-lactones require 11 linear steps, significantly fewer than the number of steps required for the synthesis of, for example, the bryostatin analogues (**9**-**27**) [160]. With this approach that exploits combinatorial chemistry [161], a remarkable series of DAG-lactones has been

Boije af Gennäs G et al. Curr Top Med Chem. 2011; 11(11): 1370-92
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created, and intensive research has revealed important determinants for binding, intracellular localization and biological activity.

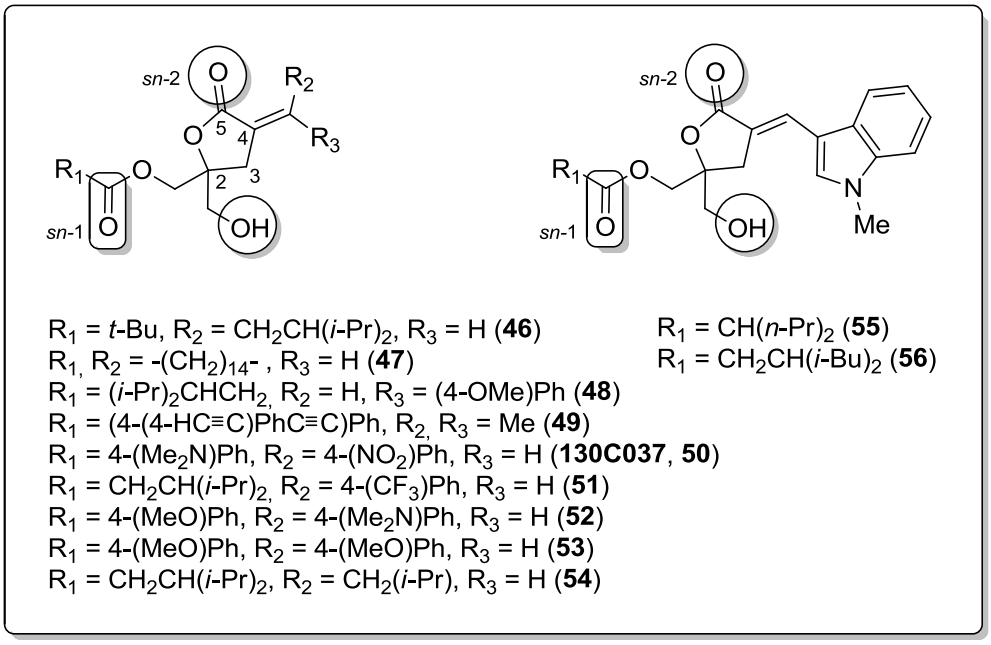

Figure 7. Structures of DAG-lactones (**46-56**). Functional groups that interact with the C1 domain are highlighted.

Structure-activity studies

Some DAG-lactones bind to PKCα with K_i values as low as 1.45 nM (e.g., the enantiomer (2*R*)-**46**) depending on the acyl and alkylidene substituents. Molecular-modeling studies performed on the DAG-lactones reveal two competing binding modes to the C1 domain [162, 163]. In the *sn*-1 mode, the carbonyl of the acyl group is hydrogen-bonded to Gly253 of the C1 domain, and the alkylidene group is pointing towards the lipid membranes. In the *sn*-2 mode, the lactone carbonyl is hydrogen-bonded to Gly253 and the alkyl chain of the acyl group is pointing towards the lipid membranes. Removing either of the carbonyl groups or substituting either with a thiocarbonyl reduces binding activity and further affects the binding of the *sn*-1 mode [162]. Bulky alkylidene groups enhance the *sn*-2 binding mode and increase the binding affinity to PKC. In addition, recently synthesized derivatives are suggested to

preferentially bind with the *sn*-2 mode [164-166], as the hydrophobic parts of the acyl group in the *sn*-2 mode are thought to interact with lipids in cellular membranes.

The following SAR results of these DAG-lactones have been reported [159]: 1) the (2*R*)-stereoisomer is the preferred one; 2) substituents at C4 increase activity and an alkylidene substituent at C4 is preferred over an alkyl substituent; 3) over a 2-fold increase in binding affinity for the *Z*-alkylidenes can be obtained when the C4-alkylidene chains are branched, with some exceptions; 4) the C3 should not be substituted due to a lack of space in the C1 binding groove [167-169]; 5) the C3 can be replaced by an oxygen with some loss of activity, but with an improved clog *P* value [170]; 6) the respective lactam and amide analogues show reduced binding affinity [171]; 7) the elimination of either the acyl or lactone carbonyl or replacing of either with a thiocarbonyl decreases activity [162]; and 8) the elongation of the C2 aliphatic acyl group decreases binding affinity [172].

The optimal clog *P* values for binding affinity seem to be approximately 5-6 [163, 173]. Recently, the research has focused on lowering the log *P* values of the DAG-lactones without overly affecting the binding affinities; for example, the macrocyclic γ-lactone (**47**, clog *P* 3.85) [173], compound **48** (clog *P* 3.62) [164] and compound **49** (clog *P* 3.24) [166] were shown to bind with K_i values of 6.1, 7 and 5.2 nM, respectively. Further SAR studies are presented in the following sections.

Selectivity of DAG-lactones among C1 domain containing proteins

Several DAG-lactones show some PKC isoenzyme selective binding [165, 174]; however, differences in binding do not necessarily translate into differences in the potency to induce biological effects [175]. It has been hypothesized that the biological outcome might depend on the differential intracellular targeting of the PKC isozymes in cells. PKC has relatively broad substrate requirements and the DAG-lactones substituted with different lipophilic side

chains may allow PKC isoenzymes to be directed to certain intracellular compartments with different lipid environments and consequently control which substrates are accessible. This issue will be discussed later in this chapter.

Despite the high level of conservation of the C1 domains, some RasGRP-selective DAG-lactones have been successfully designed. After initial screening of a series of biphenyl-substituted ligands [161], DAG-lactone **50** was further characterized [174]. The compound showed multiphasic and shallow binding curves for PKC isoenzymes with K_i values ranging from 340 nM for PKCα to 29 nM for PKCε. In contrast, for RasGRPs 1 and 3, which contain only one C1 domain, it showed monophasic binding curves with K_i values of 3.5 nM and 3.8 nM, respectively. A striking selectivity was also observed between the individual C1 domains of the PKC isoforms: the K_i values of the isolated C1a and C1b domains of PKCδ were 2780 and 1.78 nM, respectively, and the K_i values of the C1a and C1b domains of PKCα were 610 and >10000 nM, respectively. The selective binding to RasGRPs was reflected in the translocation patterns of GFP-tagged proteins and in the phosphorylation of ERK1/2 induced via RasGRP3 alone. To enhance the selectivity between PKCα and RasGRP, a set of DAG-lactones with heterocyclic moieties as α-arylidene substituents were designed [176]. In addition, the α-heteroarylidene moieties were combined with lipophilic-branched acyl chains to replace the aromatic acyl moieties for optimal activity. With this approach, K_i values of 0.18 and 0.72 nM for RasGRP3 (compounds **55** and **56**, respectively) and selectivity over PKCα by 165-fold and 119-fold, respectively, could be achieved. Interestingly, the binding selectivity decreases when the acyl group is changed to a more branched one, which is the opposite of the result usually obtained with DAG-lactones. In addition, the *E*-heteroarylidenes showed better binding affinities to PKCα and RasGRP3 compared with the respective *Z*-isomers.

Boije af Gennäs G et al. Curr Top Med Chem. 2011; 11(11): 1370-92
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Effects on cell proliferation

Several DAG-lactones and their derivatives have shown antiproliferative and proapoptotic activity. The DAG-lactone **46**, showing K_i values of 5.4 nM for PKCα and 1.6 nM for PKCδ [171], exhibited proapoptotic activity in prostate cancer LNCaP cells in a PKCα-dependent manner [177]. The effect of the enantiomer (2*R*)-**46** [160] on apoptosis was mediated by the down-regulation of ATM kinase, which is a component of the DNA damage surveillance and repair system, and led to the induction of ceramide synthesis, thus allowing DNA damage-mediated apoptosis [178]. The compound (2*R*)-**46** also sensitized prostate cancer LNCaP and CWR22-Rv1 cells to radiation-induced apoptosis. The DAG-lactone **51** reduced ATM levels even lower than the derivative (2*R*)-**46** [175]. Both of the ligands ((2*R*)-**46** and **51**) also potentiated the radiation effect *in vivo*, where they completely inhibited prostate tumor growth in Swiss nude mice and prevented a rise in serum PSA levels [175, 178].

DAG-lactones with polar groups in the 3-alkylidene chains were synthesized to improve water solubility [179]. In most cases, the modifications had little effect on binding affinity, and ligands with good PKCα-binding affinity (K_i = 3.2-5 nM) also showed significant antitumor activity against colon cancer and leukemia cell lines.

Induction of α-secretase activity

A series of branched lactones was created using the potent DAG-lactone **46** [160] as a lead structure, and the derivatives were then tested for their potential as α-secretase activators [180]. The subnanomolar binding affinities of the derivatives to PKCα and their lipophilicity correlated with the induction of α-secretase activity, which was measured as the amount of sAPPα secreted in human APP695-transfected W4 neuroblastoma cell line. The DAG-lactone (2*R*)-**46** induced a dose-dependent enhancement of the α-secretase activity and was more potent than the racemic **46** and PDBu. The enantiomer (2*R*)-**46** was further developed in

order to form it into a more drug-like structure with reduced lipophilicity [175]. Even if the analogues **52** and **53** showed weaker binding affinities to PKCα (K_i values of 887 and 332 nM, respectively, compared to 1.45 nM of the compound (2*R*)-**46**) and had >2 orders of magnitude reduction in the clog *P* values, they still increased α-secretase activity at a 1 μM concentration to 154% and 162% above the untreated cells (100%), while the template lactone increased it to 177%. Based on these results, it was concluded that good binding affinity to PKCα does not exclusively predict the effect on α-secretase activity.

HIV-1 eradication

Latent HIV-1-infected T-lymphocytes impose a risk of relapse and hamper the results of antiretroviral therapy. An emerging idea is to use agents that activate these latent reservoirs of the viruses by activating viral gene expression in combination with the conventional therapy for complete eradication of the virus pool. Because PKC is known to regulate HIV-1 transcription through several mechanisms, activation of PKC could result in beneficial effects in anti-HIV-1 therapy.

DAG-lactones **46** and **54**, showing subnanomolar K_i values for PKCα (2.9 nM and 15.1 nM, respectively [171, 181]), induced the expression of the HIV-1 marker p24 antigen in ACH-2 cells, a latently infected T-cell line, and sensitized the cells to an anti-HIV-1 immunotoxin [182]. These ligands also induced a similar set of genes involved in T-cell activation as well as viral gene expression in PBMCs derived from HIV-1-infected patients. Because the compounds were structurally different but still showed similar biological effects, it was hypothesized that compounds **46** and **54** could favor different binding modes in the C1 domain.

Boije af Gennäs G et al. Curr Top Med Chem. 2011; 11(11): 1370-92
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Correlation between ligand structure and biological response

The extensive research on DAG-lactones has produced a great amount of knowledge about the determinants affecting the binding of the ligands to the C1 domain. However, the definite SAR studies are hampered by the multifaceted interactions of the C1 domain-ligand complex with the lipid bilayer. Because the incorporation of the lipid bilayer into the binding model is difficult, the Blumberg and Marquez groups took the indirect approach of synthesizing chemical libraries, where the synthesized compounds had different combinations of the acyl and alkylidene side chains. As a result, the concept of "chemical zip codes" was introduced to explain how subtle differences in the ligand structure could translate into different biological responses by directing the activated enzyme complex to different subcellular sites [175]. It was concluded that this concept could form the basis for therapeutic strategies targeting specific pathways.

Studies performed on DAG-lactones with different rigid rod substituents at the acyl position [165, 166] and rigid rods with different termini [183] have further helped to elucidate the mechanisms of membrane interactions of the ligand-enzyme complex, the kinetics and duration of PKC translocation, and the concomitant pattern of the biological responses such as the phosphorylation of ERK1/2, the induction of IL-6 secretion, the inhibition of cell proliferation, the induction of cell differentiation [166], and apoptosis [165]. It has also become clear that in addition to high binding affinities and appropriate clog *P* values, the nature of the acyl groups are equally important in influencing the membrane interactions and the concomitant biological responses [165, 166, 183].

Boije af Gennäs G et al. Curr Top Med Chem. 2011; 11(11): 1370-92
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ANTHRACYCLINE DERIVATIVES

Anthracyclines, such as doxorubicin (DOX, **57**), are chemotherapeutic agents that are obtained from *Streptomyces peucetius* and *S. coeruleorubidus* (Figure **8**) [184]. The anthracyclines exert their antitumor effects mainly via interference with a DNA-topoisomerase II interaction during DNA replication [185]. DOX intercalates into DNA with rings B and C between the adjacent base pairs of DNA and the sugar moiety located in the minor groove of DNA [186]. This stabilizes the DNA-topoisomerase-complex, and the DNA replication and cell division are halted. However, tumor cells are able to develop resistance to DOX. To overcome DOX resistance and to reduce DOX-induced cardiotoxicity, a novel semisynthetic anthracycline-derivative AD 198 (**58**) was synthesized [187]. It only weakly binds to DNA and has little inhibitory activity on topoisomerase II [185], therefore having a different mechanism of action from the conventional anthracyclines.

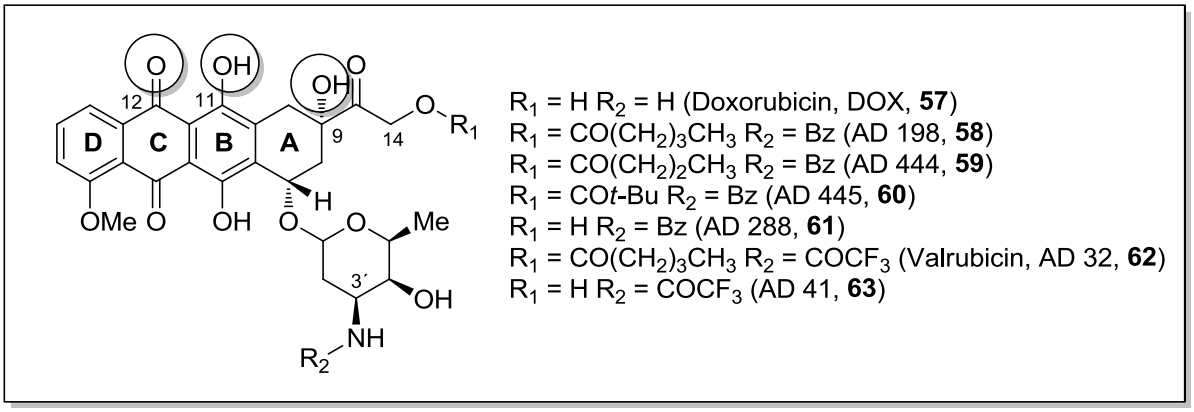

Figure 8. Structures of anthracycline derivatives (**57-63**). Functional groups that interact with the C1 domain are highlighted. In addition to these, the carbonyl of the R_1 group is thought to be involved in binding to the C1 domain.

Structure-activity studies

Roaten and co-workers showed that AD 198 targets the C1 domain of PKC [188]. By using the crystal structure of the PKCδ C1b domain, they found three theoretical binding models in which AD 198 binds in the groove of the C1b domain but less deeply than the phorbol esters (**3**). The authors proposed a binding model in which Ser240 and Gln257 bind to the C9 hydroxyl and Gly253 binds to the C12 carbonyl and C11 hydroxyl, which is also hydrogen-bonded to Leu251. The C14 valerate and C3´ N-benzyl group were thought to play a similar role to the acyl groups of phorbol esters in binding to C1 domains, and the positively charged amino group was suggested to be at least partially responsible for the translocation to the nuclear membrane. Furthermore, the valerate group of AD 198 increases the lipophilicity and causes the molecule to localize in the perinuclear region [189]. In addition, among different C14 acyl-substituted derivatives synthesized, the compounds containing 4-5 carbons (e.g., AD 198, AD 444 (**59**) and AD 445 (**60**)) were found to be most promising [190]. At the molecular level *in vitro*, AD 198 was shown to inhibit PKC activity and to compete with [^{3}H]PDBu binding to rat brain PKC, PKCδ, and the C1b domain of PKCδ and β2-chimaerin [190]. AD 198 has also been reported to cause translocation of PKCα and PKCδ to the membrane fraction in murine myeloid cells [191]. Interestingly, DOX and AD 288 (**61**), the principal AD 198 metabolite, are both incapable of inhibiting PKC activity or competing for [^{3}H]PDBu binding [190].

Effects on cell proliferation

AD 198 is effective *in vitro* against several cancer cell lines including multidrug resistant human ovarian and breast carcinoma cells [192], mouse and human leukemia cells [193, 194] and HeLa cervical cancer cells [195]. Additionally, its anti-tumor activity has proven to be superior to that of its parent compound DOX and is not influenced by anthracycline-

resistance in vivo in mice [196]. The pharmacokinetics of AD 198 have been characterized in rats [197], and *in vivo* it is hydrolyzed to AD 288, which lacks the C14 valerate group and has a pharmacological profile similar to that of DOX [190].

The cytotoxic mechanism of AD 198 is at least partially associated with its ability to activate PKC isoenzymes. It activates PKCδ, triggering the mitochondrial apoptotic cascade [191]. The apoptotic pathway involves PKCδ-dependent phosphorylation and the activation of phospholipid scramblase 3 [195, 198], which is involved in phosphatidylserine translocation to the outer leaflet of the plasma membrane during apoptosis. AD 198 also activates PKCε, which leads to cardioprotection *in vivo* [199] – further distinguishing it from cardiotoxic DOX. In addition to PKC, AD 198 has been reported to target several other cellular targets. For example, it inhibits mitochondrial palmitoyltransferases [200] and RNA polymerase [201]. However, the roles of these other targets in relation to the anti-cancer activity of AD 198 have not been clarified.

Another anthracycline derivative, valrubicin (AD 32, **62**) [193], has similar PKC-inhibitory properties as AD 198 [202], and the mechanism of action of its valerate-free metabolite AD 41 (**63**) can be compared to that of AD 288 and DOX [189]. Valrubicin was reported to be safe in a phase I trial with advanced gynecological malignancies [203] and has thereafter been approved in the US for topical treatment of bladder cancer [185].

Although not primarily developed as C1 domain ligands, AD 198 and AD 32 are two promising candidates as cancer therapeutics among C1 domain-targeting compounds. The parent compound DOX has been used in the clinic for >30 years and is still one of the most widely used cancer chemotherapeutics on the market. The different mechanism of action of AD 198 and AD 32 provides a possibility to bypass drug resistance due to reduced topoisomerase II activity – a common problem related to anthracycline therapy.

Boije af Gennäs G et al. Curr Top Med Chem. 2011; 11(11): 1370-92
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CALPHOSTIN C

Calphostin C (**64**) [204] is a polycyclic aromatic perylenequinone that was described in the 1980s as a potent PKC-selective inhibitor isolated from the fungus *Cladosporium cladosporioides* (Figure **9**) [205]. It inhibits most PKC isoforms at nanomolar concentrations, and its inhibitory effects include both the inhibition of phosphotransferase activity and the inhibition of phorbol ester binding to the C1 domain [205, 206]. Its activity against PKC was later found to be light-dependent [207] and to involve the irreversible oxidative inactivation of PKC [208]. Deletion analysis of PKC confirmed that the binding site of calphostin C is the C1 domain, although the C2 domain also seemed to play a role [206]. The inactivation of PKC by calphostin C requires cofactors (PS + Ca^{2+} or PS + PMA) even with constitutively active PKC mutants [206], suggesting that it targets the active conformation of PKC at the cell membranes. Among non-PKC phorbol ester receptors, calphostin C has been shown to inhibit diacylglycerol kinase [209] and to bind to the C1 domains of the chimaerins, unc-13, and RasGRP [72, 210, 211].

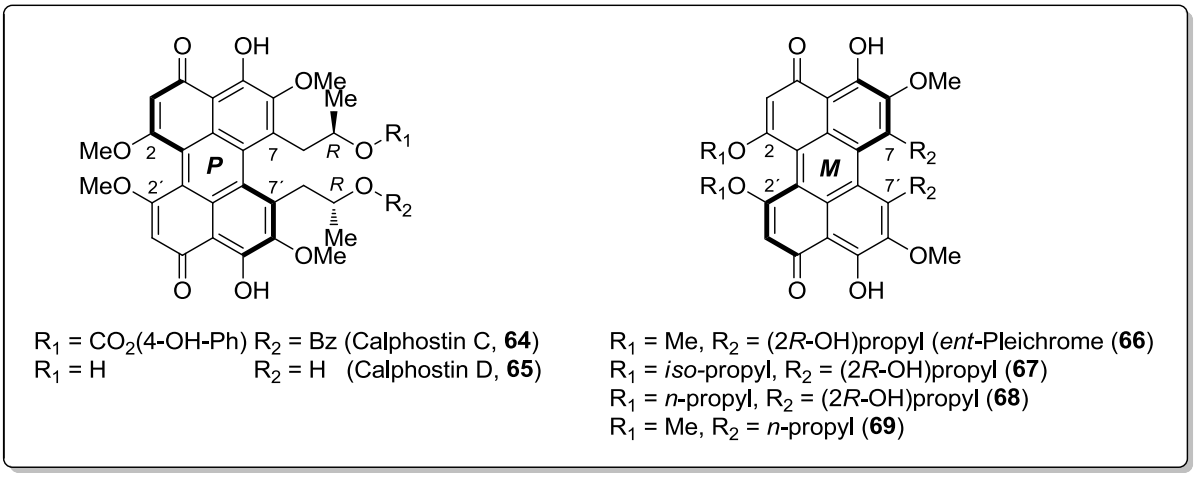

Figure 9. Structures of calphostins (**64-65**) and *ent*-pleichrome derivatives (**66-69**).

Structure-activity studies

The total synthesis of calphostin C is laborious and requires 12 steps [212, 213]. Morgan and co-workers used *ent*-pleichrome (**66**, an atropisomer of calphostin D (**65**)) and the crystal structure of the PKCδ C1b domain when designing new perylenequinone derivatives [214]. The docking studies showed an unfilled hydrophobic pocket in the protein, where the C2,C2´-methoxy groups were pointing; thus, the corresponding bisisopropyl and bis-*n*-propyl derivatives (compounds **67** and **68**, respectively) were synthesized and tested for binding. Both compounds showed increased binding affinities compared to **66** (IC_{50} values of 0.8, 1.5 and 3.5 μM, respectively). Among the calphostins, higher binding affinities can be obtained with more hydrophobic groups at C7 and C7´ [215]. Substitution of the 2-hydroxypropyl groups at C7 and C7´ of **66** with *n*-propyl groups resulted in better binding, the obtained IC_{50} value of derivative **69** was 0.4 μM. [214] Furthermore, 16- to 28-fold higher binding affinities could be obtained with the *M*-atropisomers compared to the *P*-atropisomers.

Effects on cell proliferation

Calphostin C has been shown to have proapoptotic effects in numerous cancer cell lines such as cervical carcinoma [216], prostate cancer [217], and glioma [218]. Calphostin C-induced cytotoxicity in breast cancer cells was reported to be accompanied by cytoplasmic vacuolization [219]. Calphostin C-induced cytoplasmic vacuolization that originates from endoplasmic reticulum (ER) stress precedes apoptosis in several different cell lines (glioblastoma, pancreatic carcinoma and breast carcinoma cell lines) [220]. However, the effect was not mediated by PKC. Indeed, there is a growing body of evidence suggesting that calphostin C-induced cytotoxicity is mediated through other mechanisms than PKC, including a general increase in reactive oxygen species (ROS) [216], the destruction of nuclear lamin B1 [216], the disassembly of Golgi apparatus [221, 222], and the direct

inhibition of phospholipase D [223]. Although suggested as a candidate for photodynamic cancer therapy [224, 225], there are few or no reports describing the effects of calphostin C in *in vivo* models of cancer.

OTHER C1 DOMAIN LIGANDS

Phorbol esters (**3**) are the most widely studied PKC activators, but due to their tumor-promoting properties, their potential in drug development is poor. However, prostratin (12-deoxyphorbol-13-acetate, **70**) and DPP (12-deoxyphorbol-13-benzoate, **71**) are non-tumor-promoting members of the phorbol ester family with no hydroxyl/acyl group at C12 (Figure **10**) [226, 227]. Recently, the Wender group developed a five-step synthesis to prostratin and its derivatives starting from readily available phorbol (**7**) [226]. Prostratin inhibits *de novo* HIV-1 infection by PKC-dependent down-regulation of HIV receptors at the surface of target cells [228, 229]. It also induces reactivation of HI viruses in latently infected cell lines, which then renders the viruses susceptible to highly active antiretroviral therapy (HAART) [230, 231] and opens the possibility of complete eradication. However, high doses or prolonged treatments with prostratin may not be well tolerated in humans [231]. Subsequently, a series of phorbol 13-*O*-monoesters were shown to be effective in latent virus reactivation and to induce PKC translocation patterns typical for non-tumor promoters [227].

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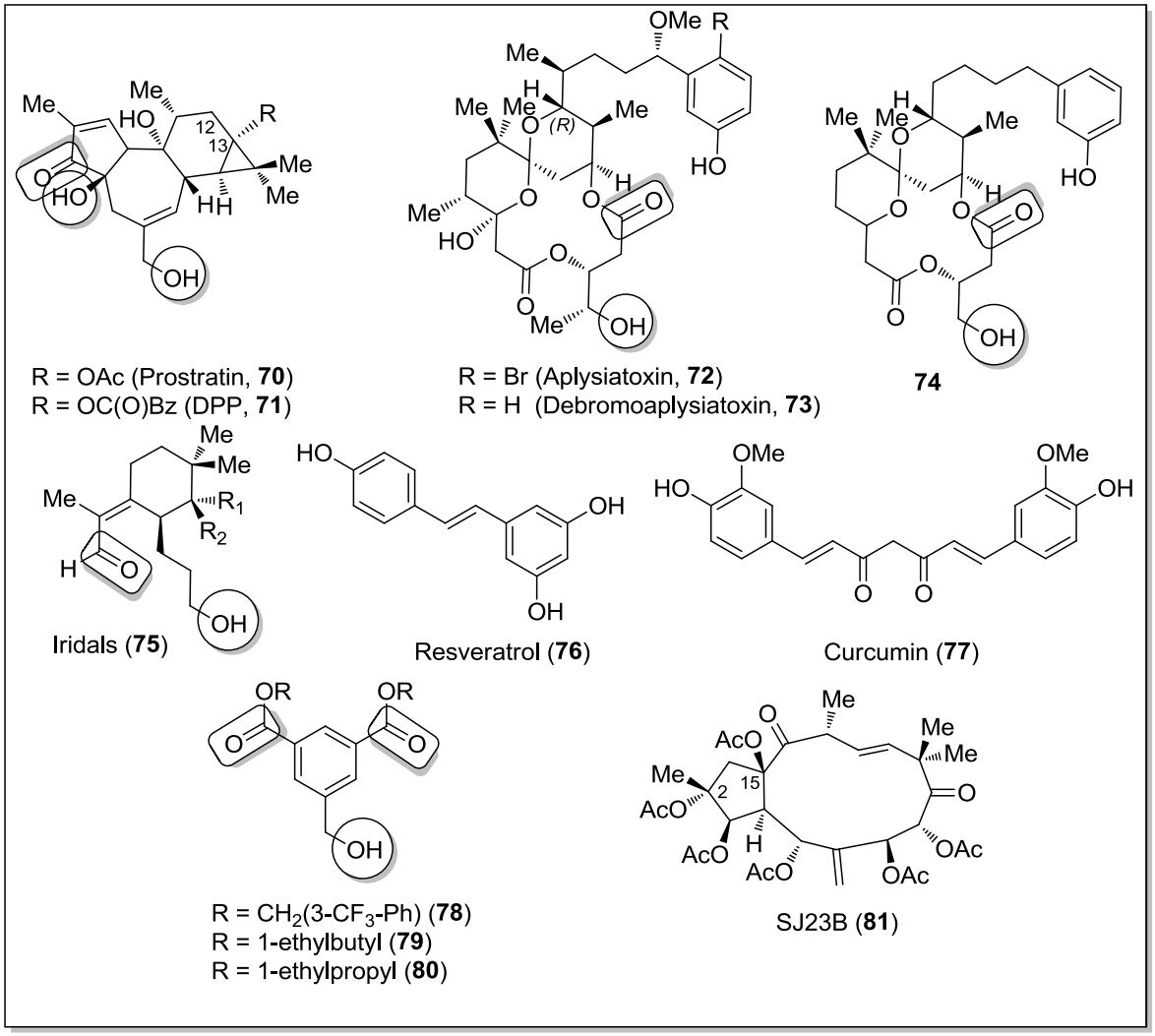

Figure 10. Structures of prostratin (**70**) and other derivatives (**71-81**). Functional groups that interact with the C1 domain are highlighted.

The marine spiroketals aplysiatoxin (**72**) and debromoaplysiatoxin (**73**) can be isolated from the sea hare *Stylocheilus longicauda* (Figure **10**) [232]. These complex compounds are tumor-promoters [233] and are structurally related to bryostatins (**9**-**10**) and other C1-binding compounds. They compete with [^{3}H]PDBu for binding to its receptors [234]. Recently, a simpler, non-tumor-promoting aplysiatoxin analogue (**74**) was developed [235]. It showed bryostatin 1-like activity with a K_i value of 15 nM to PKCδ and anti-proliferative properties in several cancer cell lines. However, the synthesis of the analogue (**74**) requires 22 steps.

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The iridal type triterpenoids (**75**) are another class of natural products (derived from *Iris* sword lilies) that bind to the C1 domains and activate PKC (Figure **10**) [236, 237]. Two iridals were shown to bind to RasGRP3 and PKCα with K_i values between 16-84 nM, showing modest selectivity (6-15 times) for RasGRP [238]. Furthermore, these compounds showed anti-tumor activity in several cell lines including A2780 and K562 human tumor cell lines as well as in the NCI (US) 60-cancer cell line screen. Recently, the first total synthesis of an iridal (**75**, R_1 = Me, R_2 = homofarnesyl) was performed, requiring over 20 steps [239].

Other nature-derived C1 domain ligands include resveratrol (**76**) and curcumin (**77**) (Figure **10**). Resveratrol is a polyphenol derived from red grapes and a variety of other plants [240] that has been shown to inhibit PKC activity (IC_{50} 2 μM) and compete with phorbol esters for binding to the C1 domains of PKCα [241]. Resveratrol has been extensively studied in cancer models [242], and in some cases its activity has been dependent on PKC [243, 244]. However, it targets various other molecules, which contributes to its anti-proliferative, anti-inflammatory, and cardioprotective properties [240]. Curcumin is a natural polyphenol found in *Curcuma longa* and consumed daily by millions of people as a spice. It binds to nPKC C1b subdomains with an affinity comparable to PMA [245]. Curcumin inhibits $A\beta_{40}$ aggregation *in vitro* and reduces amyloid plaque burden in animal models of AD [246, 247]. Recently, curcumin was shown to induce PKCδ degradation and enhance spatial learning in rats [248]. Based on its ability to suppress phorbol-induced matrix metalloproteinase-9 expression, curcumin has also been suggested to have therapeutic potential in inhibiting invasiveness and angiogenesis of brain tumors [249]. As with resveratrol, curcumin has pleiotropic cellular effects that are independent of PKC activity [250]. Due to its anti-inflammatory, antioxidant

and anti-cancer properties, curcumin is in clinical trials for a variety of diseases such as cancers, irritable bowel syndrome, type II diabetes and AD [101].

A novel group of synthetic C1 domain ligands, the 5-(hydroxymethyl)isophthalates (**78**-**80**) were recently shown to possess the same pharmacophores as the phorbol esters (**3**) and DAG (**2**) (Figure **10** and **3**) [251]. These isophthalate derivatives were designed by structure-based approach and synthesized in only four steps. They bind to PKCα and PKCδ with sub-micromolar affinities (K_i values ranging from 210 to 920 nM) and regulate PKC-dependent ERK1/2 phosphorylation in living cells. In addition, isophthalate **78** was shown to induce apoptosis in HL-60 cells but not in Swiss 3T3 fibroblasts [252]. Interestingly, the same side chains as in isophthalates **78** and **79** could be found among the compounds showing the best binding affinities in the DAG-lactone compound library [175].

A jatrophane diterpene SJ23B (**81**, Figure **10**) isolated from *Euphorbia semiperfoliata* [253] has shown a potential PKCα-mediated antiviral effect on HIV-1 infection and activation of latent HIV-1 gene expression, being 10-fold more potent than prostratin (**70**) [254]. Furthermore, it is not toxic and it does not induce cell transformation, suggesting that it lacks tumor-promoting activity. So far, no PKC-binding data has been provided. Studies with other jatrophane diterpenes revealed the importance of the acetoxy groups at C2 and C15 for the activity in HIV-1 models.

CONCLUDING REMARKS

There is exhaustive evidence suggesting that PKC isoforms could be useful therapeutic targets in different pathological disorders and an equal amount of research carried out with the aim of modulating PKC activity with different molecular entities. However, to date, no

PKC-targeted drugs have been approved for clinical use. The major reasons for the absence of C1 domain-binding drugs are the apparent inefficacy of some agents (e.g., bryostatin 1) in clinical trials and the toxicity profile of others (e.g., prostratin). In addition, some potential agents have not reached the point in the drug development pipeline for *in vivo* testing despite intensive *in vitro* research (DAG-lactones).

Regardless of the shortcomings of bryostatin 1 in cancer clinical trials, the new analogues with better selectivity might provide a solution to the toxicity and efficacy problems. Bryostatin 1 will soon be tested for its safety and efficacy in AD patients but the adverse effects especially in muscles might still restrict its use. However, bryostatin 1 could still be useful in the treatment of cerebral ischemia because the effective doses of bryostatin 1 in rats were within the doses tolerated by humans.

In a recent study, cultured colon cancer cells developed resistance to the antiproliferative effects of ingenol-3-angelate and other PKC-modulating agents [255]. The researchers speculated that the acquired resistance to PKC modulators could explain the failure of the clinical trials in patients with cancer. Therefore, in addition to the careful selection of the patients, the protocols of clinical trials should include the examination of the effects of the trial agents on PKC isoform expression/activity in tumor cells in order to get a better picture of the effects of each treatment.

The clinical trials of topical treatment of actinic keratosis with ingenol-3-angelate look very promising and the compound will probably soon reach the market. Developed by the pharmaceutical companies Peplin and Leo Pharma Ltd, ingenol-3-angelate is also being studied for possible systemic administration to treat leukemias and for an intracavitary formulation to treat bladder cancer. This is one of the stories where the centuries-old folk remedy will be refined into a clinically approved drug.

Most of the C1 domain-binding compounds are derived from nature. The plant family Euphorbiaceae seems to especially be an endless source of pharmacologically active secondary metabolites. Various marine organisms have also been a valuable source of PKC-binding compounds. However, quite often, the isolation of the compound is very laborious and the yields are extremely small. Total synthesis of the natural products is often unfeasible due to their complex structures, but the work with several natural products such as bryostatins has proved that the pharmacophores (of the natural products) may be simplified and translated into more drug-like structures that also have shorter synthesis routes.

Rational design of C1 domain-binding compounds is a reasonable alternative to the natural products, although it still faces great challenges. The crystal structure of the whole PKC enzyme still waits to be solved. Furthermore, the lipid membrane in which the C1 domain-ligand complex is partly buried is difficult to include in the binding model. However, the work carried out with the DAG-lactones has greatly improved the knowledge of the crucial determinants for the ligand-protein-membrane interaction and the concomitant biological responses. Taking into account the specific structural features of each C1 domain in their whole-protein context, including the amino acid residues crucial for ligand recognition [136, 256], more selective compounds could probably be designed, and these could even direct a subset of the C1 domains to desired subcellular compartments. An attractive approach is also the creation of bivalent C1 domain-binding compounds that could be targeted to tandem C1 domains with different spacing.

One important aspect in targeting the C1 domain is the possibility for the development of PKC agonists. However, PKC activators face the challenge of tumor promotion, which may restrict their use. Therefore, balancing the anti- and pro-tumor properties is necessary, and it is clear that long-term experiments are necessary.

Taken together, the development of C1 domain-targeted compounds is still going strong. With ever-increasing knowledge of the roles of C1 domain-containing proteins in different biological functions and of the crosstalk these proteins may have with each other in response to DAG signaling, the new chemical innovations and improved old ones will with no doubt provide us with new drug candidates in the near future.

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