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The Transition from a Pharmacophore-Guided Approach to a Receptor-Guided Approach in the Design of Potent Protein Kinase C Ligands

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ABSTRACT. The pharmacophore-guided approach used in the first phase of the design of novel protein kinase C (PKC) ligands was based on the study of the geometry of bioequivalent pharmacophores present in diacylglycerol (DAG) and in the more potent phorbol ester tumor promoters. A number of potent DAG lactones were generated by this approach, in which the glycerol backbone was constrained into various heterocyclic rings to reduce the entropic penalty associated with DAG binding. Based on the information provided by X-ray and NMR structures of the cysteine-rich, C1 phorbol ester/DAG binding domain, the DAG lactones were further modified to optimize their interaction with a group of highly conserved hydrophobic amino acids along the rim of the C1 domain. This receptor-guided approach culminated with the synthesis of a series of "super DAG" molecules that can bind to PKC with low nanomolar affinities. These compounds provide insight into the basis for PKC ligand specificity. Moreover, some of the compounds reviewed herein show potential utility as antitumor agents. PHARMACOL. THER. 82(2–3):251–261, 1999.

KEY WORDS. Protein kinase C (PKC), PKC isozymes, PKC activation, PKC binding, diacylglycerol, diacylglycerol lactones.

CONTENTS

ABBREVIATIONS. DAG, diacylglycerol; PDBU, phorbol 12,13-dibutyrate; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate.

1. INTRODUCTION

The protein kinase C (PKC) family of isozymes plays a pivotal role in cell signalling by functioning as central transducing elements of important pathways that regulate cell cycle progression, differentiation, and apoptosis (Nishizuka, 1992). Such signals are generated by a broad range of extracellular ligands that produce the lipid second messenger diacylglycerol (DAG) both through G-protein-coupled and tyrosine kinase-activated isoforms of phospholipase C (Berridge, 1984), as well as indirectly by phospholipase D (Shukla and Halenda, 1991). DAG binds to the C1 domain in members of the classical (α, β, and γ), as well as the novel (δ, ϵ, η, and θ) PKC isozymes activating their downstream pathways (Hodgkin *et al.*, 1998). Pharmacologically, the phorbol esters bind directly to the same C1 domain and function as potent and metabolically stable DAG surrogates

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(Ono *et al.*, 1989). Indeed, the phorbol esters bind with affinities that are 3–4 orders of magnitude greater than those of DAGs (Kazanietz *et al.*, 1992). Highlighting the important biological role of PKC, several other structural classes of natural products with potent activity as DAG analogues have also been characterized. From studies with these natural products, it is clear that different specific ligands for PKC can differentially activate PKC subpathways and thereby induce different patterns of biological responses. Thus, whereas the archetypical phorbol-12-myristate-13-acetate (PMA) functions as a potent tumor promoter, the macrocyclic lactone bryostatin 1 is in clinical trials as a cancer chemotherapeutic agent (Prendiville *et al.*, 1993; Jayson *et al.*, 1995), and 12-deoxyphorbol-13-acetate (prostratin) has dramatic antipromoting activity (Gulakowski *et al.*, 1997). Unfortunately, the difficulties in chemically manipulating these natural products have complicated efforts to understand the structural basis for their interactions with PKC, as well as the origins of their distinct biological effects.

To address these issues, we have sought to design synthetically accessible ligands for the C1 domain of PKC. Our initial objective has been to develop compounds with affinities comparable with those of the phorbol esters. These compounds will act both as probes and as tests of our understanding of the nature of ligand-C1 domain interactions. Our more long-range objective, using such structures as lead compounds, is to explore the basis for biological selectivity of PKC-targeted ligands.

Our synthetic strategy has been to exploit cyclic structures with an embedded DAG motif. Our starting point was that *S*-DAG possesses marked stereospecificity for PKC (Rando and Young, 1984), although it is substantially less potent. In contrast to an affinity of 0.4–0.8 nM for phorbol 12,13-dibutyrate (PDBU, **1**, R=COC_3H_7) (Kazanietz *et al.*, 1992; Sharkey and Blumberg, 1986), DAGs show affinities that are in the low micromolar range under the same conditions (Sharkey and Blumberg, 1986). Such low binding affinities of the DAGs may be attributable to the relative flexibility of the glycerol backbone, which, in contrast to the more rigid phorbol skeleton, may be forced to pay an entropic penalty during binding. We therefore sought ways to improve the binding affinities of DAGs by constraining the glycerol backbone into small- to medium-sized lactones. The intent was to approximate the conformation of bound DAG that would circumvent some of the energy penalty associated with entropy loss during binding. Our longer-term concept is that such structures may provide a scaffold for further functionalization to enhance affinity or selectivity.

2. THE INTUITIVE AND PHARMACOPHORE-GUIDED APPROACH TO CONFORMATIONALLY RESTRICTED DIACYLGLYCEROLS

2.1. *Five-Member Ring Lactones*

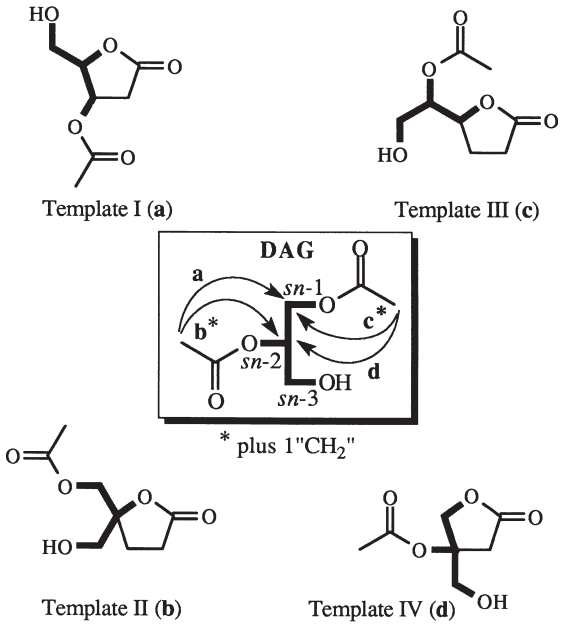
Several structure-activity studies with DAGs have shown that a range of modifications of the glycerol backbone resulted in a substantial decrease of activity. These changes included homologation of the backbone by one or two carbons (Molleyres and Rando, 1988), elimination or replacement of the *sn*-3 hydroxyl group by other moieties (Ganong *et al.*, 1986), and construction of rigid analogues of DAG through the use of carbocyclic templates (Molleyres and Rando, 1988; Rando, 1988). This cyclic template concept was also extended, without success, to mimicking the cyclohexane C-ring structure of phorbol esters (Laughton *et al.*, 1989; Kerr *et al.*, 1990). We conceived the construction of cyclic templates based on the structure of DAG itself to reduce the number of possible rotameric forms in the hope that one of the rigid rotamers would approximate the actual conformation of physiologically active DAG. As seen in Scheme 1, all the possible intramolecular cyclizations of the glycerol backbone of DAG can generate an initial library of pentonolactone templates (I–IV), which contain the glycerol moiety embedded within them. This strategy produced four basic templates: (1) 2-deoxyribonolactone (I) (Teng *et al.*, 1992; Lee *et al.*, 1993c,e, 1994d; Sharma *et al.*, 1993; Marquez *et al.*, 1994), (2) 5,5-disubstituted tetrahydro-2-furanone, also known as 4,4-disubstituted-γ-butyrolactone (II) (Sharma *et al.*, 1996; Lee *et al.*, 1996c,d); (3) 2,3-dideoxyhexono-1,4-lactone (III) (Lee *et al.*, 1996a); and (4) 2-deoxyapiolactone (IV) (Lee *et al.*, 1996a). In Templates I and II, the *sn*-2 *O*-acyl moiety is restricted into the lactone ring, whereas in Templates III and IV, it is the *sn*-1 *O*-acyl moiety that forms part of the lactone ring. Lactonization paths **b** and **c** require an additional carbon atom to complete the five-member ring structure, and for Templates I and III, additional diastereoisomers are possible, owing to the creation of a new chiral center. The major findings for these DAG-lactone templates can be summarized as follows:

(1) The creation of an additional asymmetric center by the intramolecular folding of DAG at *sn*-1 (Scheme 1, paths **a** and **c**), which leads to Templates I and III, offers little or no advantage over the open-chain DAG. The most potent compounds constructed with the chiral 2-deoxy-L-ribonolactone template showed the same affinity for PKC as DAG itself.

(2) The maintenance of just one asymmetric center by the intramolecular folding of DAG at *sn*-2, according to path **b** (Scheme 1, Template II), resulted in a large entropic advantage (10- to 100-fold higher binding affinity) over the open-chain DAG molecule. The most potent compounds constructed with this template had affinities for PKC in the 10–20 nM range. The use of this template alters the stereochemical preference from *S* to *R*, relative to DAG.

(3) The maintenance of just one asymmetric center by the intramolecular folding of DAG at *sn*-2, according to path **d** (Scheme 1, Template IV), resulted in compounds with indistinct stereochemical preference, no entropic advantage, and loss of binding affinity.

In view of these observations, it was concluded that the suitability of five-member ring γ-lactones for the construc-



SCHEME 1.

tion of constrained DAG surrogates follows the sequence II >> I ~ III > IV.

2.2. *Higher Order Lactones*

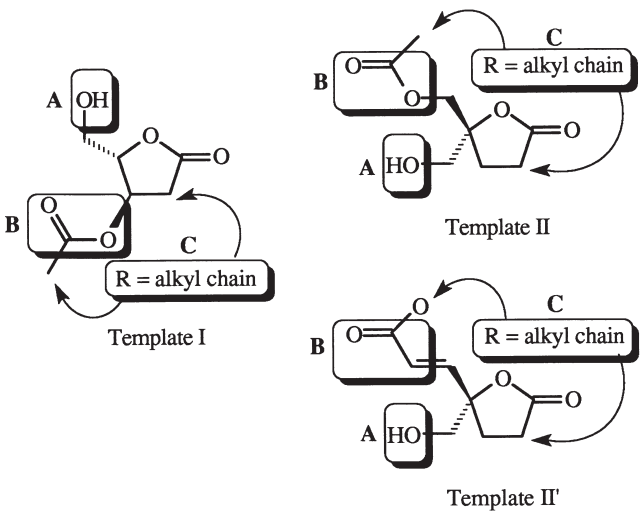
The use of six- and seven-member lactones for the construction of DAG analogues was extended only to the higher homologues of Template I (Lee *et al.*, 1993b, 1994a,b). This is because, chronologically, Template I was the first promising template discovered. Although an increase in ring-size by one carbon did not enhance binding, receptor discrimination between the optical antipodes of δ-lactones (2,3-dideoxy-D- and L-glucono-1,5-lactone) was five times greater than for the corresponding γ-lactones (Lee *et al.*, 1993b). Additionally, a more effective discrimination between isomeric α-alkylidene-δ-lactones (*Z*- and *E*-isomers) contrasted with the more modest difference between the same geometrical isomers built on the smaller α-alkylidene-γ-lactones (Fig. 1) (Lee *et al.*, 1993c, 1994a). The conclusion appears to be that the δ-lactone template provides ligands with enhanced binding specificity for PKC, and that similar higher homologues using Template II deserve to be investigated in the future.

PKC binding affinity for the larger ϵ-lactones decreased relative to the stereochemically equivalent γ- and δ-lactones. This seems to indicate that a critical ring-size of five or six is required for optimal binding (Lee *et al.*, 1994b). These findings, however, are limited to small- and medium-size lactones, where the ester linkages in the ring would exist in the *s*-cis conformation. Although we have not studied larger lactone templates, several macrocyclic DAG *bis*-lactones built by connecting the ends of the two lipophilic *sn*-1 and *sn*-2 chains have been reported (Wender and Cribbs, 1992). This operation allowed the two esters to relax to their more stable *s*-trans conformation, which, in essence, restricted the mobility of the DAG backbone. Although a full report has never appeared, the authors report K_i values for inhibiting binding of PDBU in the range of 14 nM to 3.5 μM. The recognition elements of aplysiatoxins have also been incorporated into a macrolactone, but with less impressive results (Kong *et al.*, 1991).

2.3. *Isosteric Group Replacement of Pharmacophores*

Besides the lactone carbonyl, three distinct pharmacophores common to these lactone templates can be identified: the primary alcohol function (A region), the ester function (B region), and the lipophilic alkyl chain (C region) (Scheme 2). For Template I, the A region allowed very little variation. Even though the higher alcohol homologue did show a small increase in binding (Sharma *et al.*, 1993), the presence of an additional hydroxyl group on the extra carbon (2-deoxy-D-gulonolactone analogue) resulted in a 20-fold loss in binding affinity (unpublished). The B region tolerated some minor alterations. It was found that the hydrogen-bonding capacity of the ester function could be reasonably maintained by the ether function and, to a lesser extent, by the transposed ester [RC(O)O versus ROC(O)] (Lee *et al.*, 1994d). However, the reverse ester [$RCH_2C(O)O$ versus $ROC(O)CH_2$] was equipotent to the normal ester (Lee *et al.*, 1994d). In the latter case, only the oxygen moves from one side of the carbonyl to the other, which suggests that the correct position of the carbonyl pharmacophore is very important for optimal binding. The chemical nature of this carbonyl pharmacophore is also very critical, since the corresponding ketone showed a 6-fold reduction in binding (Lee *et al.*, 1994d).

For Template II, the A region does not appear to tolerate any variations from the simple CH_2OH function. Changing the alcohol from primary to secondary [CH_2OH versus $CH(CH_3)OH$], which makes this site stereochemically equivalent to the secondary alcohol function of two other classes of potent PKC ligands, the bryostatin and the aplysi-

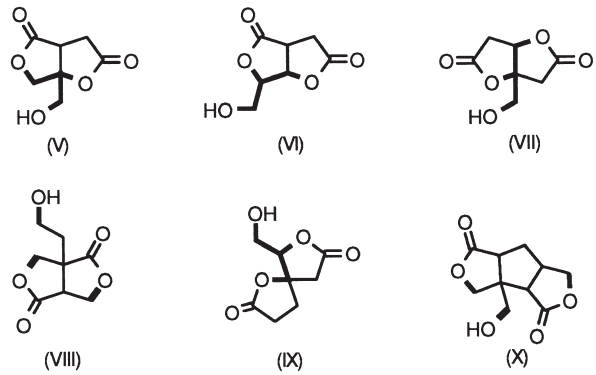


SCHEME 2.

atoxins, caused a 2-fold loss in binding affinity (Lee *et al.*, 1997). Several one-carbon higher homologues investigated with this template were 30- to 100-fold less potent than the parent compounds (unpublished). The B-region was also quite sensitive to changes. The same isosteric replacements tried on Template I, plus the exchange of the ester for an amide, resulted in net losses in binding (unpublished). Even the ether and the reverse ester isosteres showed, respectively, a 65-fold (unpublished) and 3.5-fold loss (Lee *et al.*, 1996c) in binding affinity. These results indicate that small deviations from an ideal disposition of pharmacophores in Template II are less well tolerated. However, the small 3.5-fold loss experienced by the "reversed ester" in Template II was nicely recovered by changing the ethane bridge into an ethylene bridge (Template II′), which restored the conformational bias of the "normal ester" that results from the *gauche* interaction between the two sp^3 ester oxygens (Morimoto *et al.*, 1991). The bio-isosteric equivalence of Templates II and II′ was successfully exploited for the construction of quite potent DAG lactones. Additionally, use of the "reversed ester" Template II′ helped overcome the rapid racemization experienced with the "normal ester" Template II (Lee *et al.*, 1996c). Ketone and sulfonyl functions were investigated on the B region of Template II′. The ester to ketone exchange caused only a 2.8-fold reduction in binding (unpublished). Initially, encouraging results with the $C_nH_{2n+1}SO_2$ group as an isosteric replacement of the $C_nH_{2n+1}CO_2$ group in Template II prompted the synthesis of the series of compounds where each –O–C=O (ester or lactone) was replaced by –O–SO_2 (sulfonate or sultone). However, with the exception of the first compound (i.e., *n*-$C_{14}H_{29}SO_2$ in the B region of Template II), binding affinities were lower (Marquez *et al.*, 1998). Despite their disappointing affinities, these compounds were instrumental in developing a model that attempted to explain the manner in which DAG lactones are inserted into the membrane and bind to PKC (Marquez *et al.*, 1998).

2.4. bis-γ-Butyrolactones as Rigid Analogues of Diacylglycerol

Additional constraints were imposed on the DAG backbone by extending the glycerol moiety into two fused γ-lactone rings (Scheme 3). With these rigid *bis*-γ-butyrolactone templates, multiple orientations of connected or disconnected glycerol backbones were explored by the use of perhydrofuro[3,4-*b*]furan (Templates V and VI) (Lee *et al.*, 1992, 1993d, 1996b), perhydrofuro[3,2-*b*]furan (Template VII) (Lee *et al.*, 1993a), perhydrofuro[3,4-*c*]furan (Template VIII) (Lee *et al.*, 1998), and 1,7-dioxaspiro[4.4]nonane (Template IX) (Lee *et al.*, 1994c). Additionally, one example of a *bis*-γ-lactone system separated by a spacer cyclopentane ring was investigated (Template X); however, this compound showed less binding affinity for PKC than all the contiguous *bis*-γ-lactone systems (Lee *et al.*, 1995). Consistent with the stereochemical preference for the *R* configuration in mono-γ-lactones, *bis*-γ-butyrolactone templates with the embedded (*R*)-DAG produced the more effective ligands. However, despite the expected entropic advantage provided by the additional ring plus the excellent correlation of the centers of mass of equivalent oxygen atoms between these templates and phorbol (Wang *et al.*, 1994), these molecules suffered a substantial loss in binding affinity. This loss perhaps was due to the incorrect orientation of the oxygen lone pair electrons that prevented the formation of effective hydrogen bonds with the receptor (Wang *et al.*, 1994).



SCHEME 3.

2.5. Optimization of the Lipophilic Alkyl Chain (Hydrophobicity, Bulk, and Orientation)

During the initial investigations with Templates I, II, and II′, the alkyl chain was maintained constant and equivalent in lipophilicity to myristic acid (tetradecanoic acid). This selection was made because our chosen DAG standard was glycerol-1-myristate-2-acetate (Teng *et al.*, 1992). For Template I, an important parabolic correlation between log $1/K_i$ and log (water solubility), which peaked at an optimal length around C_{14} for a straight hydrocarbon acyl chain, was demonstrated (Marquez *et al.*, 1994). As the chain length increased beyond C_{14}, affinity for PKC decreased. This was probably due to nonselective binding or to an increase in the stability of the lipid bilayer, resulting from the more extensive interdigitation between alkyl groups on the side chain and the membrane lipids. It was also demonstrated that disruption of this tight molecular association by the introduction of a "kink" on the lipophilic chain improved affinity, even if the total number of carbons increased beyond C_{14} (Marquez *et al.*, 1994). This "kink" was produced either by branching or by the introduction of a cis double bond in the middle of the chain, as in the case of oleic acid ($\Delta_{8,9}$). This result suggested that orientation of the side chain can also play an important role in modulating affinity for PKC. It was later shown that a different orientation of the alkyl chain could be induced from the α-position of the lactone ring through the synthesis of *E*- and *Z*-isomeric α-alkylidene lactones. Although in some cases the potency differences between the *E*- and *Z*-isomers were modest (2- to 3-fold, generally favoring the *Z*-isomer), the α-alkylidene lactones constructed with Templates I and II produced

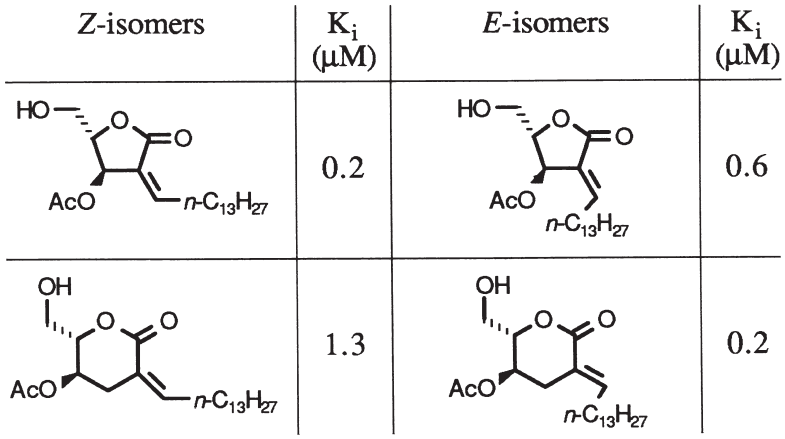


FIGURE 1. *Z*- and *E*-isomers of α-alkylidene-γ- and δ-lactones.

ligands that showed one order of magnitude better binding affinity than the equivalent templates lacking this functionality (Sharma *et al.*, 1993, 1996, Lee *et al.*, 1996c,d). A similar 10-fold increase in binding affinity was observed for 6-member α-alkylidene δ-lactones, although in this case, the *E*-isomer was 6-fold better than the *Z*-isomer (Fig. 1) (Lee *et al.*, 1993b).

2.6. *The Best Lead Compounds*

The pharmacophore-guided approach reduced the potency gap between phorbol esters and DAGs by approximately two orders of magnitude. The increase in binding affinity from glycerol-1-myristate-2-acetate (K_i ca. 1 μM) to the potent DAG lactones (K_i ca. 10 nM) was realized in two independent steps: (1) cyclization to the appropriate lactone Template II and (2) tethering of the hydrophobic alkyl chain as an α-alkylidene substituent to the lactone. Each step provided a ca. 10-fold increase in binding affinity relative to DAG (Fig. 2).

The selection of the correct enantiomer for Template II was done prior to embarking on a total synthesis of both enantiomers. As a first step, simple (*S*)- and (*R*)-DAG diacetates were modeled to fit the phorbol ester pharmacophores (Wang *et al.*, 1994; Lee *et al.*, 1996d). For this comparison, the critical functional elements that were considered to be responsible for PKC recognition of the phorbol esters [C-20 (OH), C-3 (C=O), and C-9 (OH)] were used (Nakamura *et al.*, 1989). The latter two appear to function as hydrogen bond acceptors, while the primary alcohol at C-20 functions as a hydrogen bond donor. Correspondingly, in DAG, the two ester carbonyls behave as hydrogen bond acceptors and the primary alcohol as a hydrogen bond donor. The conformation of (*S*)-DAG demanded by the phorbol ester pharmacophores was found to be a stable and low energy conformation only 4 kcal/mol above the global energy minimum (Lee *et al.*, 1996d). On the other hand, the conformation of (*R*)-DAG demanded by the phorbol ester pharmacophores was a high energy conformation 10 kcal/mol above the global energy minimum (Lee *et al.*, 1996d). Even though (*R*)-DAG, in principle, could interact with PKC through this conformation, the resulting system would have little or no free energy gain since the binding energy would be counterbalanced by the large conformational penalty. This would explain why PKC binds exclusively to (*S*)-DAG (Rando and Young, 1984).

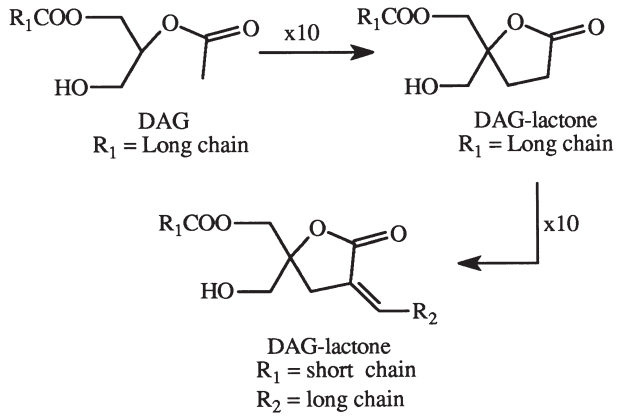
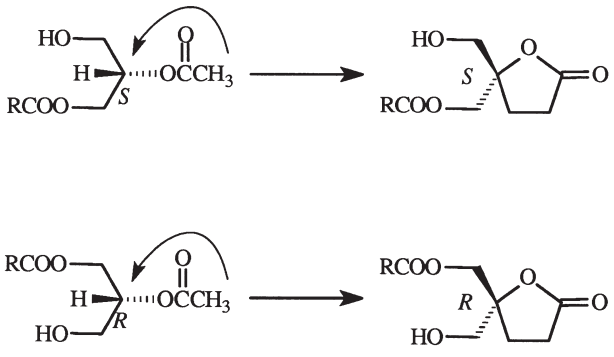


FIGURE 2. Sequence of structural changes in DAG that led to the potent DAG lactones.

For the lactones built with Template II, which conceptually could be generated from (*S*)- and (*R*)-DAG, the situation was reversed (Scheme 4). Cyclization of (*S*)-DAG into the (*S*)-lactone disrupted the desired orientation of the pharmacophores, whereas (*R*)-DAG in the energetically disfavored binding conformation could be cyclized into a stable (*R*)-lactone in which the pharmacophores remained in the correct disposition for binding. On the basis on this analysis, the (*R*)-lactone template was selected for the synthesis of chiral ligands (Lee *et al.*, 1996d). Our prediction was validated by comparing the PKC binding data between racemates and ligands containing the (*R*)-lactone Template II (Lee *et al.*, 1996d). The most potent compounds built on Template II had an α-octadec-9-enylidene side chain, where the anticipated bend caused by the *cis* double bond in the middle of the alkyl chain was intended to mimic oleic acid. Oleate esters of DAG and related lactone ligands have been shown to enhance PKC binding affinities (Marquez *et al.*, 1994). Since compounds built with Template II



SCHEME 4.

underwent rapid racemization, the equivalent analogues constructed with template II′ overcame this difficulty and preserved the same strong binding affinities (Fig. 3). The direct, spatial correspondence between the essential pharmacophore groups in phorbol esters and the primary OH and two C=O groups of the DAG lactones was an essential premise of this approach, as illustrated in Fig. 4 for Templates II and II′. Thus, the resulting heptono-1,4-lactones (entries 3 and 4, Fig. 3), represent two of the most effective and stable DAG analogues conceived by the pharmacophore-guided approach. The most potent heptono-1,4-lactone Z-isomer stimulated phosphorylation of the α-pseudosubstrate peptide (a standard substrate for PKC) with an ED_{50} of 163 ± 17 nM. In primary mouse keratinocytes, the compound caused inhibition of binding of epidermal growth factor with an ED_{50} of ca. 1 μM. Inhibition of epidermal growth factor binding is a typical, indirect response to PKC activation. In contrast to phorbol esters, the compound did not induce acute edema or hyperplasia in skin of CD-1 mice. Finally, the pattern of down-regulation for PKCα and PKCδ was different from that of PMA (Lee *et al.*, 1996a).

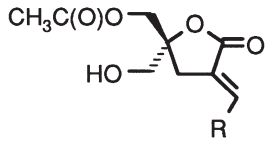
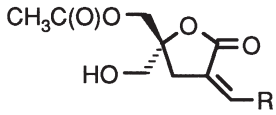
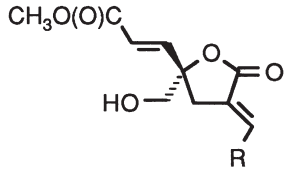
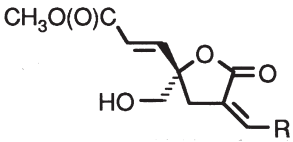
Enantiomer	K_i (nM)
	20 ± 2.9
	11 ± 0.7
	13 ± 1.3
	12 ± 0.4

FIGURE 3. Apparent K_i for enantiomeric ligands with an α-alkylidene chain as inhibitors of PDBU binding to PKCα [R=(Z)–$CH_3(CH_2)_7CH=CH(CH_2)_7$].

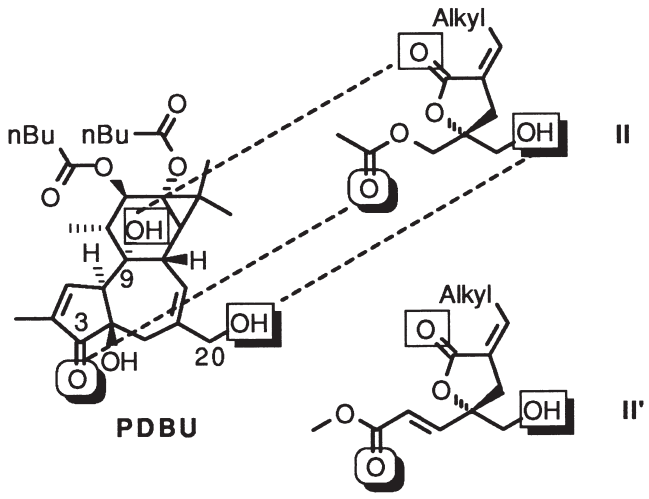
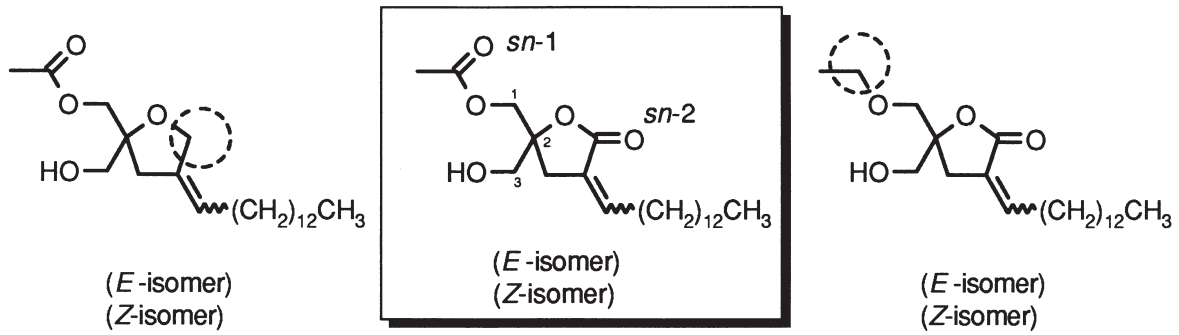


FIGURE 4. Correlation of pharmacophores between PDBU and DAG lactones (Templates II and II′) based on the pharmacophore-guided approach.

3. THE RECEPTOR-GUIDED APPROACH TO CONFORMATIONALLY RESTRICTED DIACYLGLYCEROLS

3.1. *NMR and X-Ray Structure of C1 Domains*

The potent DAG lactones designed by the pharmacophore-guided approach were based on the spatial correspondence between critical oxygen atoms in the phorbol and the DAG lactones. However, the recently available NMR structures of C1 domains of PKC (Hommel *et al.*, 1994; Ichikawa *et al.*, 1995) and the X-ray structure of phorbol-13-*O*-acetate bound to the C1b domain of PKCδ (Zhang *et al.*, 1995) presented the possibility of evaluating the mode of binding of these DAG lactones to PKC by a computer-guided, molecular docking approach. As anticipated, the X-ray structure of the phorbol/C1b complex of PKCδ confirmed the importance of hydrogen bonds involving the key phorbol ester pharmacophores C-20 (OH) and C-3 (C=O), the latter in combination with C-4 (OH) (Zhang *et al.*, 1995). However, the presumed critical C-9 (OH) pharmacophore was not found to be involved in hydrogen bonding to the protein. Instead, this OH group formed an intramolecular hydrogen bond to the C-13 carbonyl ester. Since the DAG lactones compete with the phorbol esters for the same binding site, a common mode of binding between these two classes of ligands demanded that at least one C=O group of a DAG lactone had to be involved in binding to the C1b domain of the enzyme.



SCHEME 5.

3.2. sn-*1 versus* sn-*2 Binding Modes*

Using the crystal coordinates of the C1b domain complexed with phorbol-13-*O*-acetate, the more potent *Z*-isomer (Scheme 5, box) was docked into an empty C1b domain using the program AutoDock 2.4 (Morris *et al.*, 1996; Benzaria *et al.*, 1998). This program combines a Monte Carlo simulated annealing algorithm to search the conformational space with a fast evaluation of the interaction energy. Partial flexibility was applied to the ligand by specifying nonring rotatable bonds. The search strategy consisted of performing a random walk of the ligand on the surface of the receptor, which was kept rigid. Using this procedure, the crystallographic position of phorbol-13-*O*-acetate (Zhang *et al.*, 1995) was reproduced with a root-mean-square deviation of 0.62 Å. Since DAG lactone contains an embedded glycerol backbone, the two nonequivalent carbonyl functions were identified, as in DAG, as *sn*-1 and *sn*-2. In order to reduce the number of degrees of freedom during docking, the long alkyl chain was shortened to three carbons. The docking simulation was performed 100 times. Fifty trials led to a binding mode involving the *sn*-1 carbonyl (Fig. 5, left), 43 led to an alternative binding mode involving the *sn*-2 carbonyl (Fig. 5, right), and the remaining complexes were energetically less favorable and formed outside the binding pocket. Both *sn*-1 and *sn*-2 binding modes had a comparable AutoDock scoring energy and displayed a similar network of hydrogen bonds to Thr242, Leu251, and Gly253, as observed with phorbol-13-*O*-acetate (Zhang *et al.*, 1995), with participation of the primary OH and one of the carbonyl functions. In both binding modes, one carbonyl function always remained free from hydrogen bonding to the protein.

The two binding modes revealed by the docking experiments showed an identical pattern of hydrogen bonds without regard to the disposition of the aliphatic alkyl chain. Even though the role of the aliphatic chains in DAG has been correlated mostly with partition or transport between biological phases, the two docking alternatives appear to propel the aliphatic chain in opposite directions (Fig. 5). Since the complex in the modeling does not contain a lipid matrix, it is impossible, at present, to discriminate between these two binding alternatives, based on hydrophobic interactions with the lipid membrane. Similarly, the normally higher affinity observed with the *Z*-isomer cannot be explained with this model. The study did reveal that in the two binding alternatives, one of the carbonyl esters remains uninvolved with the protein. Therefore, the individual importance of these nonequivalent carbonyl functions was tested by synthesizing DAG-lactone analogues in which a single carbonyl function was deleted (Scheme 5, broken circles). The K_i values for the compounds missing the carbonyl functions revealed a ca. 100-fold drop in binding affinity relative to the parent racemates (Benzaria *et al.*, 1998). The clear conclusion from this work was that both *sn*-1 and *sn*-2 carbonyls in the DAG lactones are essential for a strong interaction with PKC. While the results do not resolve the issue of which binding mode depicted in Fig. 5 is involved in binding to PKC, it suggests that the intramolecularly hydrogen-bonded C-9 (OH)/C-13 (C=O) motif in phorbol-13-*O*-acetate—the missing pharmacophore!—may be interacting at the same binding site as the ester carbonyl in the DAG lactones, which does not appear to be directly hydrogen bonded to the protein (Krauter *et al.*, 1996). This binding domain probably occurs within the phospholipid headgroups at the membrane interface, and since the crystal structure of the phorbol-13-*O*-acetate/C1b complex does not contain lipid, the complete picture of

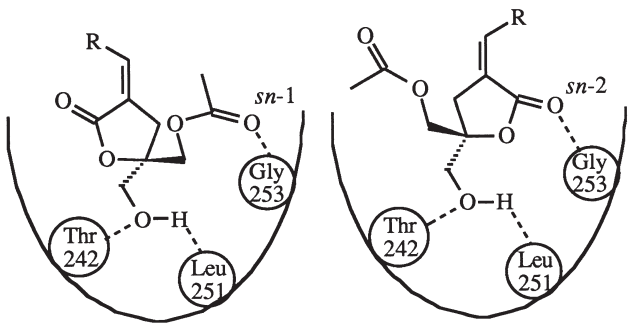
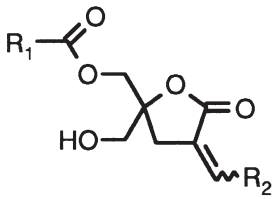


FIGURE 5. Schematic of the docking showing hydrogen bonding interactions of an α-alkylidene DAG lactone at the C1b domain of PKCδ (Benzaria *et al.*, 1998) in the alternative binding modes *sn*-1 (left) and *sn*-2 (right).



	Z-isomer	*E*-isomer
	K_i (nM)	K_i (nM)
$R_1 = CH_3(CH_2)_8$ $R_2 = CH_2CH(i\text{-}Pr)_2$	2.3 ±0.1	6.6 ±0.9
$R_1 = CH_2CH(i\text{-}Pr)_2$ $R_2 = CH_3(CH_2)_4$	7.3 ±0.5	5.5 ±0.6

FIGURE 6. α-Alkylidene and acyl branched lactones (*Z*- and *E*-isomers).

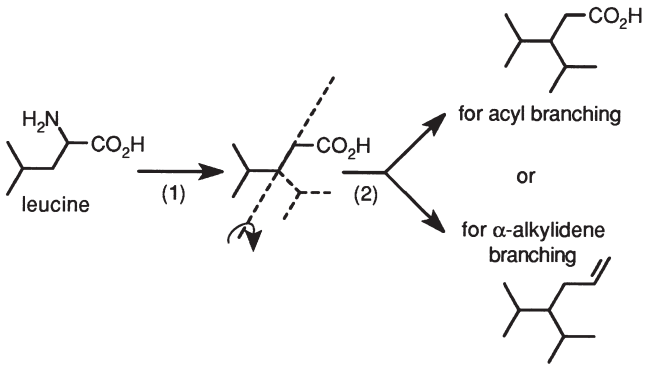


FIGURE 7. Schematic illustration of how the branched 2,3,4-trimethylpentane chain for acyl and α-alkylidene branching was generated from the amino acid leucine.

how the third pharmacophore interacts in the PKC/membrane complex remains unresolved.

3.3. *Optimization of Lipophilicity and Alkyl Chain Branching*

The crystal structure of the phorbol/C1b complex of PKCδ provided additional possibilities to design unique ligands based on the hydrophobic contacts observed between the phorbol molecule and the protein. The interaction of phorbol with a group of highly conserved hydrophobic amino acids along the rim of the C1 domain (i.e., Phe243, Leu250, Trp252, and Leu254 in PKCδ) and the spatial filling of the gap between the two β sheets are superbly fulfilled by the diterpene skeleton of the phorbol. For the DAGs, in either *sn*-1 or *sn*-2 binding modes (Fig. 5), the location of the hydrophobic amino acids provided the basis for the synthesis of branched DAG lactones designed to interact more efficiently with the hydrophobic chains and fill the gap between the two β sheets by proper manipulation of R_1 and R_2 in Template II (Fig. 6). The main idea that resulted from inspecting the nature of these hydrophobic interactions was that the long alkyl chains in the new DAG lactones had to be branched to maximize contact with the protein. The selection of an isopropyl motif for the branched chains was based on the idea of mimicking the branched amino acid Val255, and, most importantly, Leu250 and 254. The pair Leu254 and Val255 lie opposite to Leu250, which, in turn, appears sandwiched between Phe243 and Trp252. The isopropyl motif of these amino acids is also present in all of the high-affinity natural product ligands, including the phorbol esters. In designing an appropriate isopropyl motif, consideration was given to maintaining a symmetric branch, capable of reaching hydrophobic binding sites in every orientation, since these groups were to be attached to a flexible chain. For that reason, and also to achieve the required hydrophobicity, the 2,3,4-trimethylpentane chain was selected. The process of selection and construction of this chain could be envisaged, starting with the amino acid leucine. Following removal of the amino group, the generation of a symmetrical isopropyl group, as depicted in Fig. 7, produced the desired alkyl chain. This branched alkyl chain was attached to a carbonyl group for acyl branching or connected to the lactone via a methylene group for α-alkylidene branching (unpublished). The use of the branched chain as an acyl group, or as an α-alkylidene group, was designed to explore the importance of the two ends of the hydrophobic rim in the C1 domain.

3.4. *Branched Diacylglycerol Lactones*

The incorporation of an eight carbon acyl or α-alkylidene-branched chain required additional carbon atoms to be added at the other end of the molecule to reach optimal lipophilicity. The culmination of this approach for both series is shown in Fig. 6. These compounds initially were prepared as racemates for expediency, and syntheses of the corresponding enantiomers are in progress. With these branched lactones, we have achieved the highest binding affinities of any known DAG analogues. Relative to PDBU (K_i 0.4–0.8 nM) these DAG lactones are ca. 1.25- to 2.50-fold less potent, assuming that the potency of the single enantiomer is half of the racemate. This means that with relatively simple compounds that have only one asymmetric center one has practically surmounted the potency difference that separated DAGs from the phorbol esters.

4. ANTITUMOR ACTIVITY IN THE NATIONAL CANCER INSTITUTE *IN VITRO* SCREEN

Phorbol esters, and in particular PMA, are extraordinarily potent stimulators of differentiation in HL-60 human promyelocytic leukemia cells *in vitro*. Recently, patients with myelocytic leukemia who were refractory to conventional methods of therapy experienced temporary remission of disease symptoms following the administration of PMA (Han *et al.*, 1998). We became interested in seeing whether our potent DAG lactones would show antitumor activities commensurate with their potent PKC binding activity. The

results of the National Cancer Institute screening with a 60-cell line panel (Boyd and Paull, 1995) revealed specific selectivity towards leukemic cell lines. One of the acyl-branched compounds (Fig. 6, second entry), for example, had a GI_{50} of $<10^{-7}$ M against three leukemia cell lines, viz. HL-60, K-562, and MOLT-4 (unpublished). This concentration was 100-fold lower than the average GI_{50} (10^{-5} M) for the rest of the panel of tumor cells. Similar selectivities were found against a colon cell line (COLO 205) and a breast cancer line (HS 578T). Finally, the COMPARE program (Paull *et al.*, 1989), which seeks to group compounds in mechanistic categories depending on the characteristic fingerprint of the histograms against the 60 cell lines, showed a high correlation between these DAG lactones and a number of PKC activators, including some phorbol esters (e.g., prostratin, a potent inhibitor of tumor promotion), ingenol and indololactam analogues. This is a completely unbiased result that demonstrates how the combined efforts of pharmacophore- and receptor-guided approaches have produced a novel class of potentially useful antitumor DAG lactones by targeting the regulatory domain of PKC. Future biological experiments will exploit and expand on these findings.

5. FUTURE PERSPECTIVES

With the combination of pharmacophore- and receptor-guided approaches, we have designed a series of synthetically accessible ligands with affinities for PKC comparable with those of the phorbol esters. In principle, the DAG lactones have dispelled the myth that chemical complexity and pharmacological potency go hand in hand in PKC recognition and activation. The ease with which DAG lactones possessing a diverse array of side chains can be synthesized bodes well for the future use of combinatorial libraries aimed at exploiting molecular diversity for PKC and other high-affinity receptors for DAG and the phorbol esters. DAG is now known to interact with four classes of high-affinity receptors, comprising a total of 15 family members identified so far. Because of their ubiquitous distribution and extensive characterization, the diverse family of PKC isozymes represents the primary challenge. Efforts in this area will help improve our understanding of PKC isozyme function, and may uncover therapeutically useful compounds. Increasing attention is being directed at the chimaerins (Caloca *et al.*, 1997), which act on p21Rac, and RasGRP, which functions as an activator or Ras (Ebinu *et al.*, 1998). These receptors modulate complementary central signaling pathways in the cell. Hence, a library of DAG lactones will provide a useful tool to explore the multiple levels that regulate the selectivity of information flow through these receptors. Our more long-range objective will be to use lead structures that had been discovered combinatorially to explore the basis for biological selectivity of PKC-targeted ligands. These will include the differential involvement of DAG lactones in isozyme activation, proteolysis and down-regulation, cell cycle progression, apoptosis, and neoplasia.

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