Journal of Medicinal Chemistry

Volume 44, Number 25 December 6, 2001

Letters

Conformationally Constrained Analogues of Diacylglycerol. 18. The Incorporation of a Hydroxamate Moiety into Diacylglycerol-Lactones Reduces Lipophilicity and Helps Discriminate between *sn-1* and *sn-2* Binding Modes to Protein Kinase C (PK-C). Implications for Isozyme Specificity

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Received August 20, 2001

Abstract: An approach to reduce the log *P* in a series of diacylglycerol (DAG)-lactones known for their high binding affinity for protein kinase C (PK-C) is presented. Branched alkyl groups with reduced lipophilicity were selected and combined with the replacement of the ester or lactone oxygens by NH or NOH groups. Compound **6a** with an isosteric *N*-hydroxyl amide arm represents the most potent and least lipophilic DAG analogue known to date.

Introduction and Background. Membrane-generated diacylglycerol (DAG) resulting from the stimulus-initiated activation of phospholipase C induces the translocation of cytosolic protein kinase C (PK-C) to the inner leaflet of the cellular membrane.[1,2] The resulting association of PK-C with membrane phospholipids in the presence of DAG fully activates both calcium-dependent classical isozymes α, β, and γ and novel or calcium-independent δ, ϵ, η, and θ isozymes.[3,4] The transiently generated DAG, however, binds only weakly to the C1 domains of the enzyme(s), which is in sharp contrast to the well-known, high binding affinity displayed by the phorbol esters. Phorbol esters function as potent and metabolically stable DAG surrogates with the capacity to bind to PK-C with affinities several orders of magnitude greater than those of the commonly studied DAGs.[5]

Over the past few years, we have attempted to bridge the affinity gap between phorbol esters and DAGs by two independent but mutually complementary approaches.[6,7] The first approach seeks to reduce the entropic penalty associated with DAG binding by constraining the glycerol backbone into five-member DAG-lactones. The second approach involves the use of highly branched alkyl chains to improve the interaction of the DAG-lactone ligand with a cluster of conserved hydrophobic amino acids in the space between the two β sheets of the C1 domain.[8] Modeling experiments using the crystal coordinates of the C1b domain of PK-Cδ in complex with phorbol-13-acetate[8] revealed two possible binding orientations when the DAG-lactones were docked into the empty C1b domain.[7] These two apparently equivalent binding modes, *sn*-1 and *sn*-2, form a network of hydrogen bonds with amino acids Thr242, Leu251, and Gly253 identical to those formed by phorbol-13-acetate in the crystal structure of the complex, as schematically represented in Figure 1. On the basis of the two nonequivalent carbonyl moieties of the DAG-lactones, the *sn*-1 binding mode is defined as that in which the *sn*-1 carbonyl and the hydroxyl group form H-bonds with the protein. In the alternative *sn*-2 binding mode, the *sn*-2 carbonyl and the hydroxyl group are engaged in H-bonding with the protein. A prediction is that these two binding orientations can direct the hydrophobic groups (R_1 and R_2) of the ligand into rather different directions and that differences in binding affinities should reflect preferences between these two binding modes. It should be noted that molecular

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10.1021/jm0103965 CCC: $20.00
Published on Web 11/09/2001

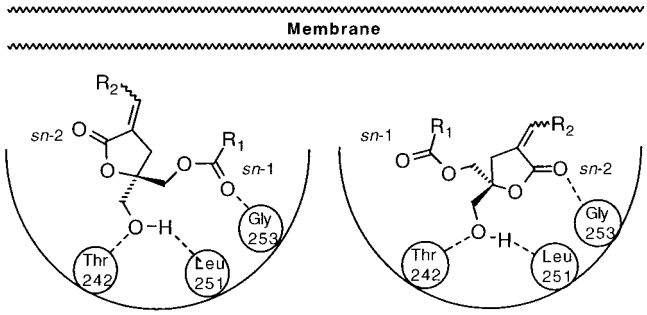


Figure 1. H-bonding interactions of DAG-lactones at the C1 domain of PK-C in alternative binding modes *sn*-1 (left) and *sn*-2 (right).

modeling is limited to examining *only* binary complexes between the C1 domain and the DAG-lactone ligands, since the phospholipid component of the ternary complex is missing in the experimentally available X-ray structure.[8]

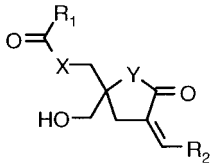
In our previous studies with DAG-lactones, we combined linear and branched R_1 and R_2 alkyl chains, but were only able to guess at the preferred binding mode. In fact, in most instances it appeared that both binding alternatives coexisted.[7] To increase our chances of designing C1 domain, isozyme-specific DAG-lactones, we felt that it was imperative to know which specific binding mode, *sn*-1 or *sn*-2, was operating.

While the role of the alkyl chain in DAGs has been principally correlated with providing adequate lipophilicity to facilitate partitioning into a lipid-rich environment, docking of the DAG-lactones into the C1 domain of PK-C revealed potentially important hydrophobic contacts with the protein that could provide the means of achieving discrimination between *sn*-1 and *sn*-2 binding modes.[7] Thus, an increase in binding affinity due to the branched alkyl chains would probably result from a combination of two factors: adequate membrane partitioning and specific hydrophobic contacts with the protein. We anticipated that by reducing the lipid dependency required for membrane distribution, the compounds would be more effectively discriminated by the protein in terms of the two possible binding alternatives (*sn*-1 versus *sn*-2), since this process of recognition would allow protein−ligand interactions to become the dominant forces.

While working with a set of DAG analogues, we recently showed a practical way to minimize lipid-dependency as a way to increase interaction of the ligand with the protein through the gradual reduction of log *P*.[9] In the present work, the application of this approach to DAG-lactones started with the progressive reduction of log *P* from the potent, symmetrically branched DAG-lactone (**1a**, Table 1).[7] We will demonstrate that this approach, coupled with other structural changes, reduces lipophilicity by more than 2 orders of magnitude without sacrificing binding affinity for PK-C. A critical change that allowed this reduction in log *P* was the isosteric replacement of the side chain ester group with an *N*-hydroxyl amide group [RC(O)O → RC(O)NOH], which lowered the log *P* to an unprecedented calculated value of 3.58, almost matching the value of the prototypic phorbol ester, phorbol-12,13-dibutyrate (PDBU, calculated log $P = 3.43$). In addition, the N-OH group provided a fourth pharmacophoric group that effectively discriminated between both *sn*-1 and *sn*-2 binding alternatives since transposition of the NOH group to replace the lactone oxygen resulted instead in a very weak ligand. This situation contrasts with compounds where both chemical groups are esters (side chain ester and lactone) which bind in a combination of *sn*-1 and *sn*-2 modes.[7] In the present work, we propose that the *N*-hydroxyl amide group is able to direct the binding of DAG-lactones into the C1 domain of PK-C exclusively in the *sn*-2 mode.

Drug Design Considerations and Biological Results. The gradual steps taken in the structure−activity analysis leading to the potent *N*-hydroxyl amide DAG-lactone **6a** are described in Table 1. For the sake of simplicity, only the geometric *Z*-isomers will be discussed in the analysis, although both *Z*- and *E*-isomers were synthesized and tested. The DAG-lactone **1a**, selected as the parent compound for this approach, was identified in a previous study as a high binding affinity ligand for PK-Cα ($K_i = 2.89$ nM) in a competition assay that measures the ability of the ligand to displace bound [20-^{3}H-PDBU] from the enzyme.[7] This molecule contains two similar branched alkyl chains, one acyl (R_1) and one α-alkylidene (R_2). The corresponding *E*-isomer **1b** was also a potent ligand for PK-Cα, but only the *Z*-isomer **1a** displayed potent and selective antitumor activity in the NCI 60-cell line in vitro screeen.[7] Since we also discovered that DAG-lactones bearing linear acyl chains were devoid of cellular activity—presumably caused by cleavage of the acyl group by cellular esterases—it was important to maintain R_1 as a highly branched alkyl group. Thus, the first step toward reducing log P involved trimming four carbons off the alkyl group of **1a** to give the smallest possible and highest branched R_1 group (i.e., pivaloyl). Relative to compound **1a**, such change lowered the lipophilicity of **2a** by almost 2 log *P* units without compromising binding affinity, which was only reduced ca. 3-fold. Since the pivaloyl group was considered an ideal small group stable toward hydrolysis, it was maintained constant. To recover the 3-fold loss in potency caused by the smaller pivaloyl group, the branched α-alkylidene (R_2) group was enlarged by two additional carbons to give compound **3a**. This compound equaled **1a** in potency and was still 0.86 log *P* units lower (more hydrophilic). Support for the pivaloyl group as the smallest and most effective acyl group was provided by compound **4a**, which contained the original larger branched acyl group (R_1) of **1a** and the same α-alkylidene (R_2) chain of **3a**. This change was not advantageous since the resulting compound (**4a**) was not only less potent but more lipophilic. Having reached what appeared to be the ideal set of branched acyl and α alkylidene chains in compound **3a**, we turned our attention to the ester moiety. Changing the ester moiety into an amide (X = O → NH) was desirable, since for the same set of branched acyl and α-alkylidene chains the log *P* was reduced by ca. 1 log *P* unit. Unfortunately, the affinity of compound **5a** for PK-C plummeted. However, when the amide hydrogen in compound **5a** was replaced by a hydroxyl group (X = NH → NOH), potency was restored, and, relative to the initial DAG-lactone **1a**, the new compound (**6a**) was 2.3 log P units less lipophilic! This change has produced the most potent and least lipophilic DAG analogue known to date.

Table 1. Structure−Activity Analysis of log *P* and K_i as a Function of Size of the Acyl and α Alkylidene Chains[a]



no.	R_1	R_2	X	Y	*E*/*Z*	log *P*	K_i (nM)
1a	$CH_2CH(i\text{-}Pr)_2$	$CH_2CH(i\text{-}Pr)_2$	O	O	*Z*	5.89	2.89 ± 0.2
1b	$CH_2CH(i\text{-}Pr)_2$	$CH_2CH(i\text{-}Pr)_2$	O	O	*E*	5.89	2.70 ± 0.4
2a	$(CH_3)_3C$	$CH_2CH(i\text{-}Pr)_2$	O	O	*Z*	4.04	8.32 ± 0.7
2b	$(CH_3)_3C$	$CH_2CH(i\text{-}Pr)_2$	O	O	*E*	4.04	11.25 ± 0.7
3a	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	O	O	*Z*	5.03	2.90 ± 0.4
3b	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	O	O	*E*	5.03	4.51 ± 0.5
4a	$CH_2CH(i\text{-}Pr)_2$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	O	O	*Z*	6.88	6.87 ± 0.6
4b	$CH_2CH(i\text{-}Pr)_2$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	O	O	*E*	6.88	4.46 ± 0.4
5a	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	NH	O	*Z*	3.95	429.7 ± 5.5
5b	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	NH	O	*E*	3.95	1260 ± 90
6a	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	NOH	O	*Z*	3.58	5.42 ± 0.3
6b	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	NOH	O	*E*	3.58	4.81 ± 0.4
7a	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	O	NH	*Z*	5.01	4330 ± 280
7b	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	O	NH	*E*	5.01	4840 ± 330
8a	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	O	NOH	*E*	4.64	10430 ± 450

[a] log *P* was calculated according to the fragment-based program KOWWIN 1.63.[10]

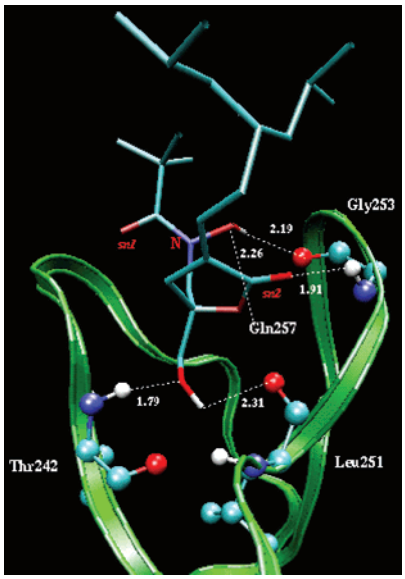


Figure 2. Docking results showing H-bonding interactions of **6a** with the C1b domain of PK-C*δ* exclusively in the *sn*-2 binding mode.

In addition, the extra NOH provides for an effective discrimination between *sn*-1 and *sn*-2 binding modalities, must likely by engaging in specific H-bonding with the protein (vide infra). When a similar change in the lactone ring was implemented by replacing the lactone oxygen for NH (**7a**,**b**) or NOH (**8a**), the resulting ligands behaved extremely poorly and displayed high K_i values (Table 1). The implications of these changes in terms of the two alternative binding modes are discussed below.

Computational Modeling and Discussion. The use of AutoDock version 2.4[11] for the docking of **6a** into the empty C1b cavity of PK-C*δ*[8] revealed the existence of a single binding mode (Figure 2). In this unique *sn*-2 binding mode, the binding of the lactone carbonyl (*sn*-2) to the NH group of Gly253 appears reinforced by a strong H-bond coming from the *sn*-1 NOH group. There is also an additional H-bond between the oxygen of the NOH group and the amide carbonyl of Gln257 that is absent in the phorbol ester PK-C*δ* complex.[8] This single binding mode of **6a** represents a significant step toward achieving isozyme selectivity, since knowing exactly what orientation the ligand prefers at the binding site will help select appropriate acyl or α-alkylidene groups designed to interact specifically with key hydrophobic amino acids of the various isoforms. As anticipated, the N-OH lactam **8a** failed completely to dock at the expected active site, confirming the validity of the model.

Recent results by Kazanietz et al.[12] show that although no differences in the activity of **6a** toward isozymes α and *δ* were observed in in vitro inhibition constants (K_i) from competitive binding curves against PDBU, as well as in its capacity to stimulate PK-C-catalyzed phosphorylation of the α-pseudosubstrate peptide,[13] the compound was exquisitely selective in directing the translocation of these isozymes specifically to either the cellular membrane (PK-Cα) or the nuclear membrane (PK-C*δ*). The consequences of this specific translocation are discussed in a companion paper describing the strong apoptosis inducing activity for **6a** in LNCaP prostate cancer cells.[12] Since molecular modeling is limited to examining only binary complexes between the C1 domain and the ligand, the role of the membrane in influencing isozyme specificity remains unresolved. An additional structural element that could be responsible for the specific membrane interaction of **6a** is the subtle but important orientation of the α-alkylidene chain induced by the *Z*- or *E*-stereochemisty of the double bond. This structural element confirms the crucial role played by the branched hydrophobic chains as only the *Z*-isomer was found active in inducing apoptosis. Since no significant differences between **6a** and **6b** were detected in the PK-C in vitro binding assay (Table 1), this method has to be used only as a first screening step. It appears that isozyme selectivity for this class of compounds is best assessed by using PK-C isoforms tagged with green fluorescent protein to visualize cellular compartmentalization.[1,2,9,12]

Acknowledgment. This work was partially supported by Grant KOSEF 2000-2-21500-001-3 from the Korea Science and Engineering Foundation.

Supporting Information Available: Detailed discussion of the syntheses and analytical data for all target compounds presented in Table 1 are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0103965