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Synthetic Diacylglycerols (DAG) and DAG-Lactones as Activators of Protein Kinase C (PK-C)

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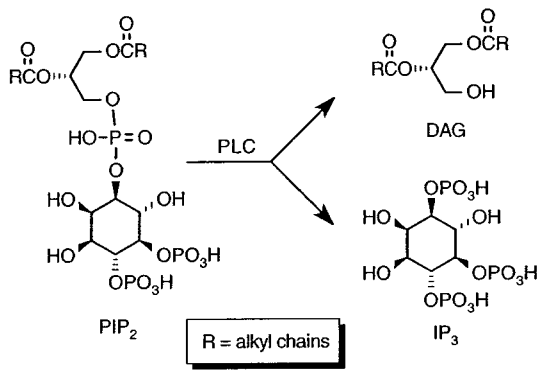
ABSTRACT

The central role of protein kinase C (PK-C) in cellular signal transduction has established it as an important therapeutic target for cancer and other diseases. We have developed a series of 4,4-disubstituted-γ-butyrolactones, which contain a constrained glycerol backbone (DAG-lactones) and behave as potent and selective activating ligands of PK-C with affinities that approach those of the structurally complex natural product agonists, such as the phorbol esters. This Account traces the design and construction of these molecules. Initially, we examined the consequences of reducing the entropic penalty associated with the transformation of a DAG into a DAG-lactone. Then, using molecular modeling to extend insights arising from the newly solved crystal structure of a C1 domain complexed with phorbol ester, we incorporated amino acid-specific branched hydrophobic chains to provide a new generation of DAG-lactones that have the capacity to bind to PK-C with low nanomolar affinity. Depending on the specific pattern of hydrophobic substitution, some DAG-lactones are able to induce selective translocation of individual PK-C isozymes to different cellular compartments, and since the specific nature of these hydrophobic interactions influences biological outcome, some of these compounds exhibit cell-specific antitumor activity. The ability to direct specific PK-C isozyme translocation with sets of structurally simple, yet highly potent molecules provides a powerful tool for engineering a plethora of molecules with novel biological functions.

1. Introduction

Our emerging understanding of the molecular events leading to cancer has focused attention on the central role played by signal transduction pathways. Prominent among these pathways are those downstream from the lipophilic second messenger *sn*-1,2 -diacylglycerol (DAG). We now understand that a major signaling system involves the receptor coupled activation of phospholipase C (PLC) activity, leading to the breakdown of membrane phosphatidylinositol-4,5-bisphosphate (PIP_2) (Scheme 1).[1] The resultant inositol-1,4,5-triphosphate (IP_3) triggers the release of calcium from intracellular stores, raising intracellular calcium levels and activating calcium-sensitive signaling pathways. The other hydrolysis product, DAG, interacts with proteins containing a so-called C1 domain, causing their activation and/or translocation to different cellular sites. Additional pathways for generation of DAG exist, such as indirect generation by the concerted action of two hydrolytic enzymes involving phospholipase D and a phosphatidic acid hydrolase. An important consequence of the existence of multiple pathways of DAG generation is that through differential modulation of DAG versus co-activators such as calcium, the cell may produce great diversity of response.

Scheme 1



The protein kinase C (PK-C) family, which constitutes the most prominent mediator of DAG signaling, contains 11 family members, divided into three subclasses. All the classes contain a C-terminal kinase domain with serine/threonine specific kinase activity and an N-terminal regulatory domain (Figure 1). The regulatory domain contains a pseudosubstrate domain (PS), which is thought to occupy the catalytic site of the catalytic domain and inhibit activity. Multiple interactions at the regulatory domain stabilize the protein in an unfolded configuration, removing the pseudosubstrate domain from the catalytic site and thereby activating the enzyme. Of the three subfamilies of PK-C isoforms, the so-called "classical" and "novel" isozymes contain twin C1 domains, which bind DAG and confer DAG responsiveness. The classical isoforms (α, β1 and β2, and γ) also possess a C2 domain responsible for calcium responsiveness, whereas the novel isoforms (δ, ϵ, η, and θ) lack the C2 domain and are not calcium regulated. In addition to DAG and calcium (for the classical isoforms), the cellular membrane represents a further important regulator with the proportion of phosphatidylserine being an important determinant of activity.

Dr. Marquez received his Ph.D. in Medicinal Chemistry from the University of Michigan in 1970. After one year of postdoctoral training at the National Cancer Institute (NCI), he worked in private industry for five years in Venezuela. He rejoined the NCI in 1977 as a Visiting Scientist and was awarded tenure in 1987 after becoming a naturalized citizen. In 2000 he became Chief of the Laboratory of Medicinal Chemistry at NCI-Frederick.

Dr. Blumberg received both his A.B., summa cum laude, and his Ph.D. from the Department of Biochemistry and Molecular Biology, Harvard University. He was Helen Hay Whitney fellow at the Cancer Center, Massachusetts Institute of Technology, and was assistant and associate professor of pharmacology at Harvard Medical School. Since 1981, Dr. Blumberg has been Chief of the Molecular Mechanisms of Tumor Promotion Section.

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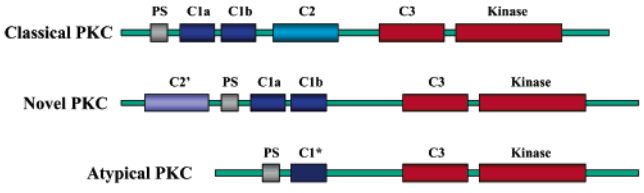


FIGURE 1. Primary structures of the PK-C subfamilies showing the various domains. The novel PK-Cs contain a homologue of the C2 domain (C2′) which does not bind calcium. The atypical PK-Cs contain a variant of the C1 domain (C1*) that does not bind DAG.

In addition to the PK-C family, five other families of proteins have been identified with C1 domains responsive to DAG. The PKD/PKC μ family represents kinases superficially similar to PK-C; however, the kinase domains are not homologous and show different selectivity. They lack a pseudosubstrate domain, show different spacing between their C1 domains, and contain a membrane interacting PH domain. The RasGRP family members contain a functional domain which activates Ras or Rap activity. The chimaerins contain a functional domain which inhibits Rac activity. Finally, the munc proteins are involved in the priming of vesicle fusion and DAG kinase functions to abrogate DAG signaling.

2. The C1 Domain as a Target for Modulation of DAG Signaling Pathways

The traditional approach for targeting an enzyme is through the development of inhibitors of the enzyme's catalytic activity. With PK-C, there has been some level of success with this approach starting with analogues of the potent natural product staurosporine.[2] However, an underlying problem with catalytic site inhibitors is the abundance of kinases encoded in the genome and the marked homology in their catalytic sites.

We have therefore explored a complementary strategy, with different advantages and challenges, namely, the design of modulators targeted to the C1 domain. This strategy has several advantages. First, the number of DAG responsive C1 domain containing proteins is much less than the number of kinases. Second, proof of principle for the utility of this approach emerged early on from the characterization of potent natural products (vide infra) directed to C1 domains. With the identification of multiple family members, it has become clear that PK-C isoforms are not interchangeable. Rather, different isoforms may be mutually antagonistic. For example, PK-Cδ is growth inhibitory in NIH 3T3 cells, whereas PK-Cα or ϵ is growth-stimulatory. Thus, rather than by inhibition of a growth stimulatory isoform through its catalytic domain, the same result may be achieved by stimulation of a growth inhibitory isoform through its regulatory domain.

Complex natural products have provided many of the initial insights into the structural features required for ligand binding and for the diversity of biological responses. These ligands include the diterpenes such as the phorbol esters, macrocyclic lactones such as the bryostatins, polyacetates such as aplysiatoxin, or indole alkaloids such as teleocidin. The abundance of structural solutions found by nature for generating high-affinity ligands speaks to the central biological role played by the PK-Cs and other C1 domain containing family members. On the other hand, their structural complexity has limited the opportunities to manipulate these structures. We therefore became interested in the potential of the much simpler structure of the endogenous ligand for the C1 domain, DAG, as a platform for ligand design.

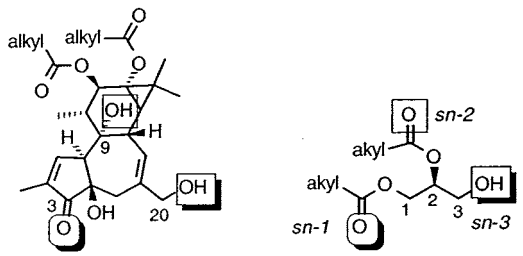


FIGURE 2. Corresponding pharmacophores on phorbol and DAG.

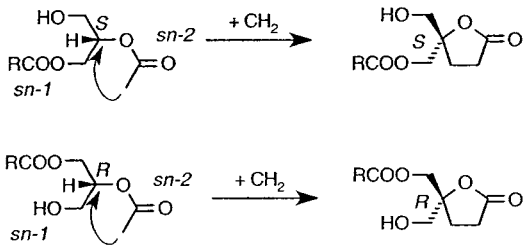
3. An Intuitive Pharmacophore-Guided Approach to Conformationally Restricted DAGs

3.1. The Selection of Five-Member Ring Lactones as DAG Templates. Before the structure of the C1 domain became available, several models attempted to correlate stereospecific hydrophilic interactions—specifically, H-bonding—between ligands and a hypothetical receptor.[3] All existing models concurred with at least a three-point pharmacophore. For DAG, carbonyls at positions C_1 (*sn*-1) and C_2 (*sn*-2) were considered H-bond acceptors and the C_3 OH (*sn*-3) a H-bond donor. Correspondingly for phorbol, the most consistent model involved the C_{20} OH as an H-bond donor, the C_3 carbonyl as an H-bond acceptor, and the C_9 OH as a donor or acceptor (Figure 2).[4] This model was later shown to be partially correct after the crystal structure of the C1b domain of PK-Cδ bound to phorbol-13-*O*-acetate was solved.[5] Despite this excellent correspondence of pharmacophores, the difference in binding affinities between DAGs and phorbol esters is at least 3 orders of magnitude in favor of the latter.[6,7]

A first step to overcome this potency gap involved the design of ligands based on cyclic structures containing an embedded DAG motif. Our starting point was the structurally simple *S*-DAG, a molecule with marked stereospecificity for PK-C binding.[8] We reasoned that the low binding affinity of DAG was attributable, in part, to the flexible nature of the glycerol backbone and the associated entropy penalty. In a recent review, we have discussed the reasons why five-member ring lactones—specifically 4,4-disubstituted-γ-butyrolactones—represent ideal glycerol templates.[9] The generation of this template involves the *sn*-2-*O*-acyl moiety of DAG joining the glycerol backbone by a pathway that requires an extra carbon atom to complete the five-member ring (Scheme 2).

3.2. The Preferred Stereochemistry for DAG and DAG-Lactones. Unlike DAG, whose stereochemical preference for PK-C is *S*, the preference for DAG-lactones is *R*. When

Scheme 2



(*S*)- and (*R*)-DAG were modeled to fit the phorbol ester pharmacophore according to Figure 2, their conformational energies were, respectively, 4 and 10 kcal/mol above the global energy minimum.[10] This explains why PK-C binds exclusively to (*S*)-DAG.[8] However, when (*S*)-DAG is converted into a (*S*)-DAG-lactone, the orientation of a key carbonyl pharmacophore changes during ring closure resulting in an inactive template (Figure 3, path a). In contrast, the cyclization of (*R*)-DAG produces no changes in the disposition of pharmacophores (Figure 3, path b). Thus, cyclization into a (*R*)-DAG-lactone cancels the energy penalty associated with the binding of (*R*)-DAG and explains PK-C's preference for the (*R*)-DAG-lactone enantiomer. This was experimentally validated after comparing the active (*R*)-DAG-lactones with their corresponding racemates (Table 1). As expected, the K_i values for (*R*)-DAG-lactones **2**−**6** were ca. half the values of the racemates. The K_i value measures the affinity of the ligand in terms of its ability to displace a bound [^{3}H-20]-phorbol ester (PDBU, Figure 2) from PK-C. The lower the K_i, the more effective the ligand.

3.3. The Nature and Disposition of the Alkyl Chains on the DAG-Lactone Template. Table 1 also illustrates the importance of the alkyl chain's disposition as a critical determinant for biological potency. Compound **1** represents the simplest racemic DAG-lactone with only one acyl group. Although small differences in affinity were observed between myristoyl [$(CH_3(CH_2)_{12}CO$] and oleolyl [$(CH_3(CH_2)_7$-$CH{=}CH(CH_2)_7CO$, *Z*-isomer] side chains in compound **1**, the transposition of the large aliphatic chain from the acyl position (*sn*-1) to the α-alkylidene position (*sn*-2), as in compounds **3**−**6**, resulted in a 5−8-fold increase in binding affinity. This operation produced a DAG-lactone (**4**) that was ca. 20-fold more potent than the equivalent 1-oleolyl-2-acetyl-sn-glycerol (K_i = 230 nM).[11] In general, the binding affinity displayed by DAG-lactones **3**-**6** and other DAG-lactones with linear alkyl chains was independent of the stereochemistry of the α-alkylidene group (Table 1). However, a consistent >2-fold increase in binding affinity was observed for the *Z*-isomers when the α-alkylidene chains were branched (Table 2).[12] Surprisingly, when both acyl and α-alkylidene chains were branched the stereochemical preferences again disappeared. It was not until the receptor-guided approach was developed that these differences were understood (vide infra).

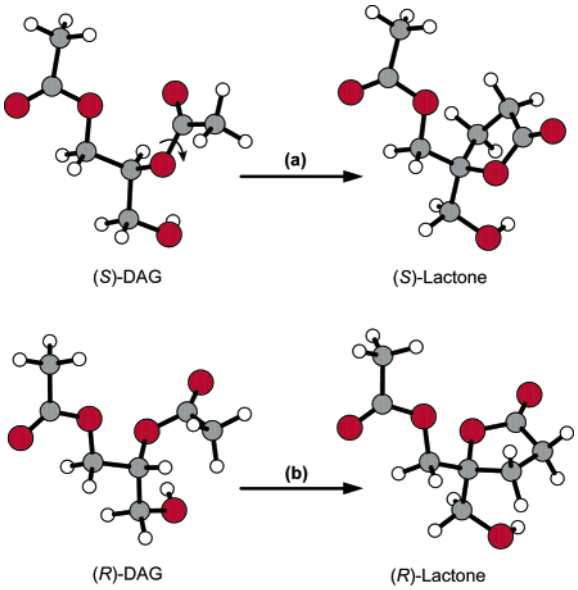


FIGURE 3. Idealized cyclizations of (*S*)- and (*R*)-DAGs.

Table 1. Apparent K_i (nM) Values for Ligands 1−6 as Inhibitors of PDBU Binding to PK-Cα

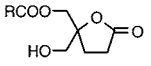
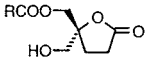
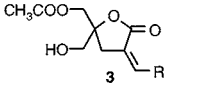
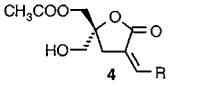
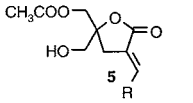
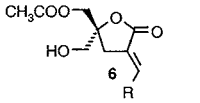
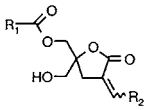
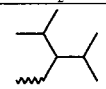
Racemate	K_i	(R)-Enantiomer	K_i
 **1**	138±24	 **2**	96±3.1
R = $CH_3(CH_2)_{12}$  **3** R	24±2.87	R = $CH_3(CH_2)_{12}$  **4** R	12±0.40
R = $CH_3(CH_2)_7CH{=}CH(CH_2)_7$  **5** R	28±2.19	R = $CH_3(CH_2)_7CH{=}CH(CH_2)_7$  **6** R	13±1.30
R = $CH_3(CH_2)_7CH{=}CH(CH_2)_7$		R = $CH_3(CH_2)_7CH{=}CH(CH_2)_7$	

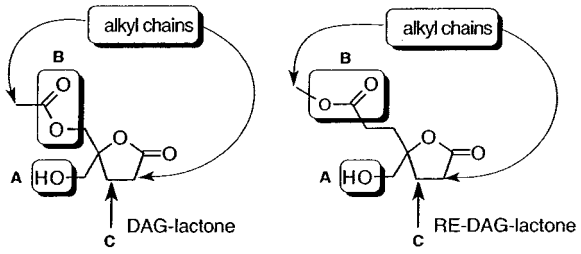
Table 2. PK-Cα Binding Affinity Ratios (*E*/*Z*) of α-Alkylidene- and Acyl-Branched Lactones (*E*- and *Z*-Isomers)



R_1	R_2	E/Z^1
C_nH_{2n+1} n = 1 → 9		2-3
	C_nH_{2n+1} n = 1 → 9	0.8-1

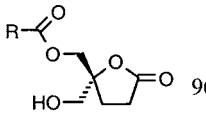
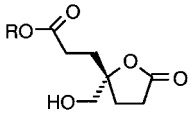
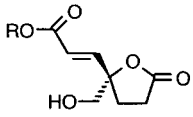
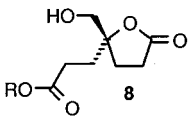
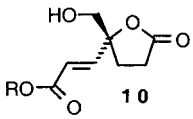
[1] Ratio of K_i values.

Scheme 3



3.4. Isosteric Groups, Additional Substitutions and Reverse Ester (RE) DAG-Lactones. The A and C regions of the template (Scheme 3) do not tolerate variations.

Table 3. Apparent K_i Values Comparing DAG-Lactones and Reverse Ester (RE) DAG-Lactones [R = $CH_3(CH_2)_{12}$]

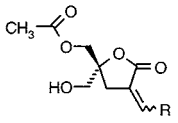
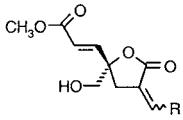
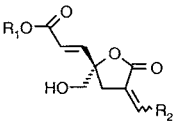
DAG-lactone	K_i (nM)	RE-DAG-lactone	K_i (nM)	RE-DAG-lactone	K_i (nM)
 **2**	96±3.1	 **7**	260 ±41	 **9**	109 ±6.7
		 **8**	27,300 ±2,500	 **10**	27,300 ±7,700

Higher homologues of the A region were 30−100-fold less potent than the parent compounds (unpublished results), and the addition of OH groups at position C_3 of the lactone (C region) significantly reduced binding affinity.[13] The B region was also quite sensitive to changes. Replacement of the ester group by a ketone or an amide produced ineffective ligands,[14] and transposition of the ester oxygen and carbonyl moieties reduced binding affinity (unpublished). Even the recently reported replacement of the ester by an *N*-hydroxyl amide [RC(O)O → RC(O)NOH][14] appears to be ineffective in light of the discovery that the structure of the alleged *N*-hydroxamate was incorrect.[15] Inasmuch as the position of the carbonyl ester appeared critical, reverse ester (RE) DAG-lactones were investigated (Scheme 3) with the intent of achieving stable compounds resistant to acyl migration and racemization of the kind observed with compounds **2**, **4**, and **6**. This approach appeared promising as the simplest RE-DAG lactone **7** was only 2.7-fold less potent than the parent DAG-lactone **2** (Table 3). This drop in binding affinity was attributed to the loss of the gauche interaction between the two sp^3 ester oxygens in the RE template. Gauche interactions between oxygens are well known to impart some conformational constrain about the single bond by favoring the gauche disposition over the intuitively favorable *ap* orientation.[16] The conformational bias of the ester branch in **2** was restored by a double bond (compound **9**), thus establishing the isosteric equivalence between RE-DAG-lactone **9** and DAG-lactone **2**. The more than 2 orders of magnitude preference for a single enantiomer (**7** vs **8** and **9** vs **10)** parallels the strict sterochemical preference already described for DAG (vide supra). The RE-DAG lactone concept was explored further with the synthesis of RE-DAG-lactones **13**−**16**, which, in contrast to **11** and **12**, were stable to racemization (Table 4).[17] In contrast to DAG-lactones, the RE-DAG template was sensitive to the stereochemistry of the α-alkylidene chain, even when it was linear (compounds **13**-**16**, Table 4). These rigid analogues probably favor a single binding mode, something that was later corroborated by molecular docking studies in the receptor-guided approach.[12]

4. The Receptor-Guided Approach to Conformationally Restricted DAGs

4.1. X-Ray Structure of C1 Domain. The pharmacophore-guided approach was based on the spatial correspondence between critical oxygen atoms in phorbol and DAG-lactones. The advent of the X-ray structure of phorbol-13-*O*-acetate bound to the C1b domain of PK-Cδ[5] allowed further evaluation of these concepts by molecular docking approaches. The crystal structure confirmed the importance of H-bonds involving the C_3 carbonyl, which in combination with the C_4 OH binds to the amide and carbonyl moieties of Gly253. The C_{20} OH accepts a H-bond from the amide of Thr242 and donates a bifurcated H-bond to the carbonyls of Thr242 and Leu251 (Figure 4).[5] Surprisingly, the critical C_9 OH was not involved in binding the protein, and instead it formed an intramolecular H-bond to the C_{13}-carbonyl ester. Considering that the role of this carbonyl has been shown to be critical,[18] it is possible that such an intramolecular motif interacts with phospholipid headgroups outside the C1 domain.

Table 4. PK-Cα Binding Affinities (K_i) and log *P* for α-Alkylidene RE DAG-Lactones (*E*- and *Z*-Isomers)

	E-isomer K_i (nM)	*Z*-isomer K_i (nM)
 R = $CH_3(CH_2)_7CH{=}CH(CH_2)_7$ **11** (*E*-isomer) **12** (*Z*-isomer)	13±1.3	12±0.4
 R = $CH_3(CH_2)_7CH{=}CH(CH_2)_7$ **13** (*E*-isomer) **14** (*Z*-isomer)	20±2.9	11±0.7
 R_1 = $CH_3(CH_2)_5$ R_2 = $CH_2CH(i\text{-}Pr)_2$ **15** (*E*-isomer) **16** (*Z*-isomer)	29.5±2.5	3.5±0.1

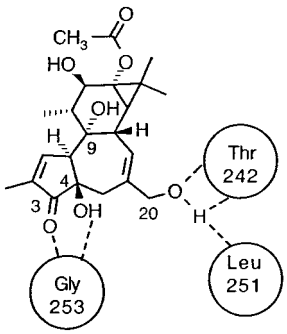


FIGURE 4. Hydrogen bond networks of the phorbol/PK-Cδ C1b complex.

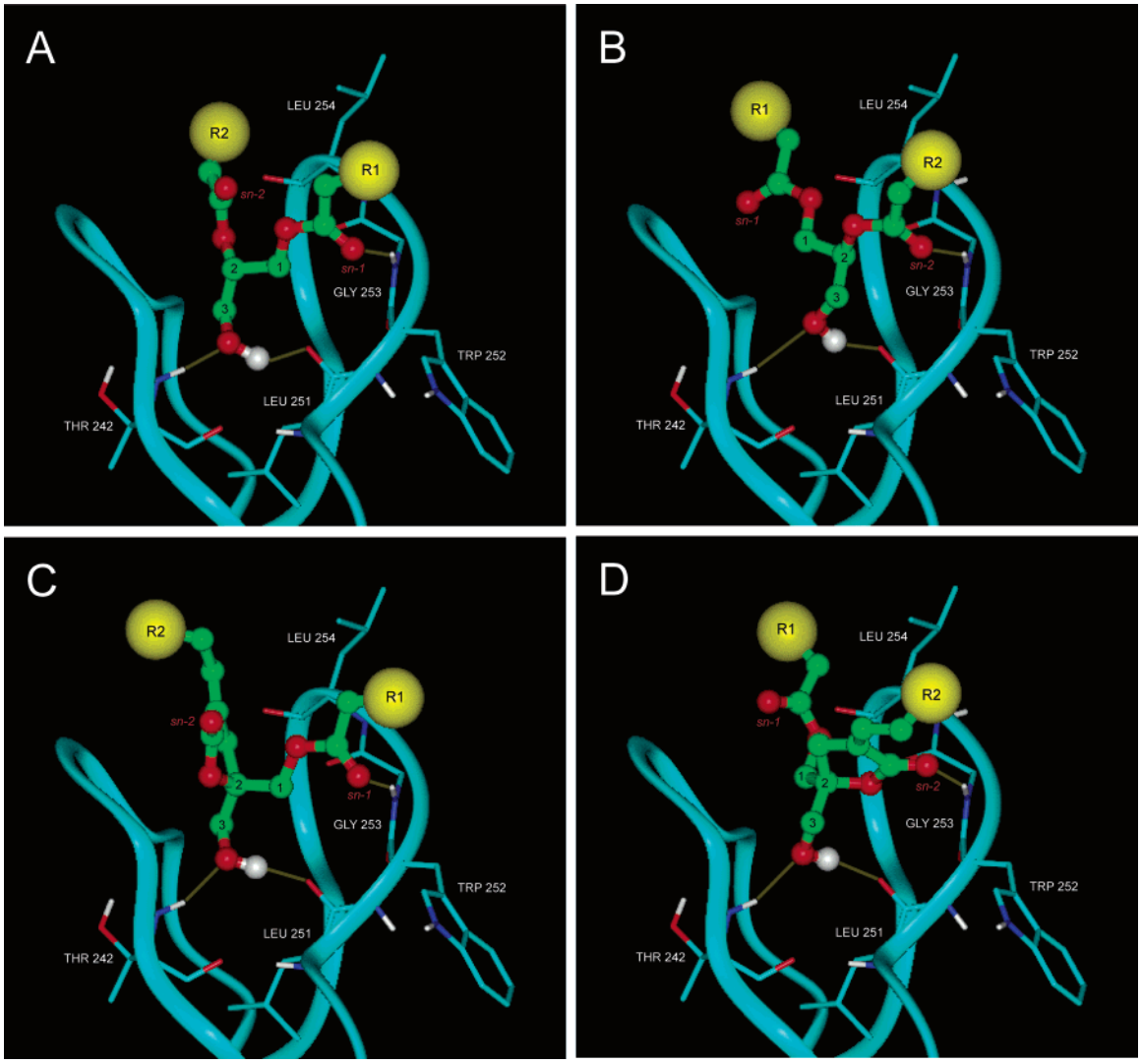


FIGURE 5. Hydrogen bonding interaction of DAG (A, B) and DAG-lactone (C, D) with the C1 domain of PK-C in the alternative *sn*-1 (left) and *sn*-2 (right) binding modes.

4.2. *Sn*-1 versus *sn*-2 Binding Modes. Docking studies with the program AutoDock 2.4[19] reproduced the crystallographic position of phorbol 13-*O*-acetate in the C1b domain of PK-Cδ.[5] With DAGs or DAG-lactones the program consistently identified two similar binding modes with an identical network of H-bonds matching those seen with phorbol, but with only one of the two nonequivalent carbonyl functions, *sn*-1 or *sn*-2, directly engaged in binding. In the case of RE-DAG-lactones, only the *sn*-2 binding mode was observed.[12] We have labeled these binding modes as *sn*-1 or *sn*-2, depending on which carbonyl binds the protein (Figure 5). Despite the lack of involvement of one carbonyl, when each was removed separately, the resulting DAG-lactones experienced a ca. 100-fold drop in binding affinity, suggesting that both *sn*-1 and *sn*-2 carbonyls are essential for a strong interaction with PK-C.[20]

4.3. Design and Construction of the Branched Alkyl Chains and Their Relationship with Lipophilicity (log *P*). Despite the nearly identical pattern of H-bonds seen in both *sn*-1 and *sn*-2 binding modes, these docking alternatives propel the aliphatic chains (R_1 and R_2) in opposite directions (Figure 5). This observation, combined with the presence of a group of conserved amino acids along the rim of the two loops of the C1b domain of PK-Cδ (Met239, Pro241, Phe243, Leu250, Trp252, and Leu254) provided a rationale to modify these aliphatic chains to optimize hydrophobic interactions in different orientations. Branched chains containing isopropyl groups were chosen to mimic branched amino acids such as Leu and Val. These chains were to be attached to either the carbonyl group (R_1) or the lactone ring (R_2) (see Table 2), and to facilitate interactions in every orientation, they were made symmetric, as in 2,3,4-trimethylpentane and 2,4,6-trimethylheptane.[12,14] Initially, lipophilicity was kept constant and equivalent to that of myristic acid (tetradecanoic acid). This selection was made because our chosen DAG standard was glycerol-1-myristate-2-acetate and because an important parabolic correlation between log $1/K_i$ and log(WS) (WS = water solubility) peaked when the straight hydrocarbon chain was 14 carbons long.[21] A similar parabolic dependence between log $1/K_i$ and the more commonly used parameter log *P* was also seen for a set of DAG-lactones bearing a combination of linear and

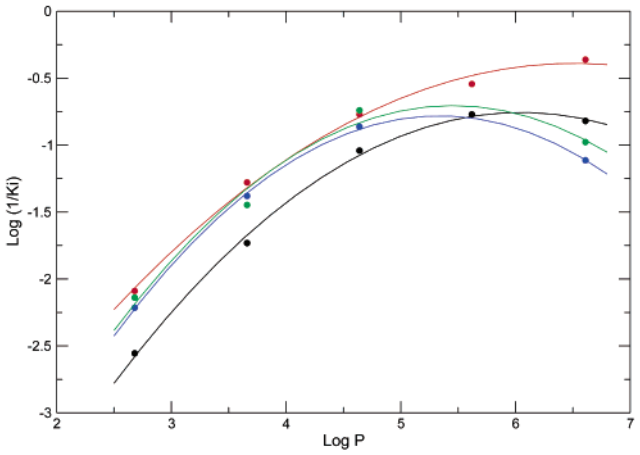
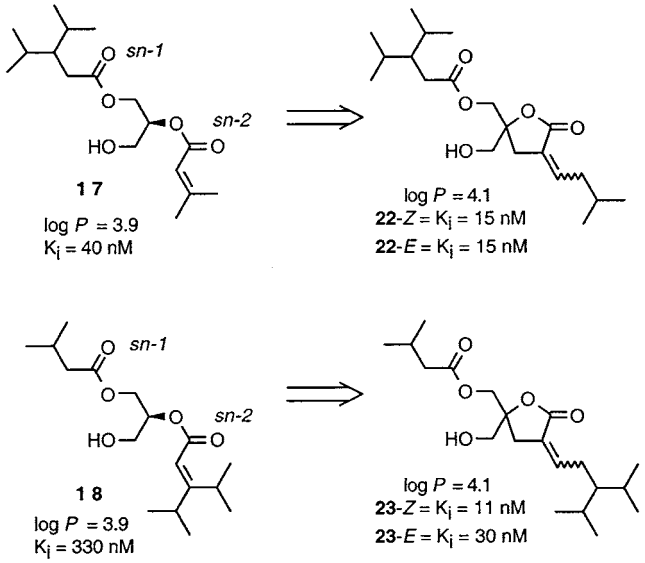


FIGURE 6. Binding affinity (log $1/K_i$) vs log *P*. The red (*Z*-isomers) and black (*E*-isomers) curves correspond to the branched α-alkylidene series, and the blue (*Z*-isomers) and green (*E*-isomers) curves correspond to the acyl branched series.

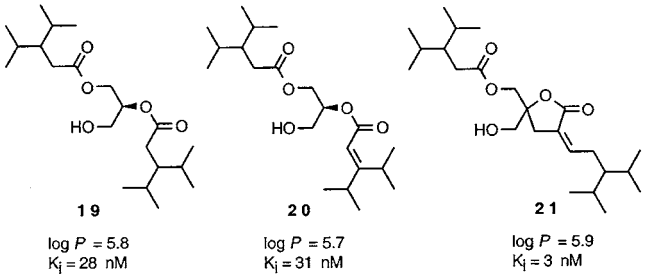
branched acyl or α-alkylidene chains (Table 2, Figure 6). The log *P* (octanol/water partition coefficient) is a measure of the hydrophobic/hydrophilic balance of the molecule and is calculated by the fragment-based program KOWWIN 1.63.[22] While the role of the alkyl chains in DAGs and DAG-lactones can be considered as principally correlated with providing adequate lipophilicity (higher log *P* values) to facilitate partitioning into the lipid-rich membrane environment, these nonspecific interactions had to be reduced to an absolute minimum in order to detect specific contacts with the protein. The use of branched chains was a good strategy toward this goal since branching lowers the log *P*.[22]

4.4. *Sn*-1 versus *sn*-2 Binding Modes in DAGs. Since the branched alkyl chains had such a dramatic impact on DAG-lactones, we decided to investigate the role of branching on DAG. This was important considering that all commonly used DAGs, such as 1,2-dioctanoyl-*sn*-glycerol (diC8), 1-oleoyl-2-acetyl-*sn*-glyerol (OAG), and 1,2-dioleolyl-*sn*-glycerol (diolein), have linear alkyl chains. On the basis of the two existing binding alternatives, which would propel the branched alkyl chains in opposite directions (Figure 5A,B), a prediction was that measurable differences in DAG binding must correlate with a preferred binding mode (*sn*-1 or *sn*-2). The log *P* of DAGs was reduced to match the log *P* of the high-affinity phorbol ligand, PDBU (log $P = 3.4$), which is lower than the log *P* of all commercially available DAGs, even diC8 (log $P = 5.3$). We identified DAGs, such as **17** and **18** (log $P = 3.9$), that were efficiently discriminated by PK-Cα by as much as 8-fold (Scheme 4). This result confirmed the ability of PK-C to discriminate between two different orientations of the side chains. The exceptional binding affinity of **17** was achieved despite a 1.4 log reduction in log *P* relative to that of diC8. The compound also activated PK-Cα with an EC_{50} of 9.5 ± 0.8 μM, compared to 16.4 ± 2.9 μM for diC8, and contrary to diC8 it effectively translocated the entire full-length PK-Cα protein to the membrane in intact cells.[23] Additional studies showed that while binding of **17** to the C1b domain of PK-Cδ was sensitive to mutations,

Scheme 4



Scheme 5



particularly of Trp252, it was less dependent on the presence of phospholipid. Whereas PDBU binding to wild-type PKC-δC1b decreased 68-fold in the absence of phosphatidylserine, the binding of **17** only decreased by 20-fold. Thus, in the absence of phospholipids, **17** bound with only 15-fold weaker affinity than did PDBU![24]

4.5. *Sn*-1 versus *sn*-2 Binding Modes in DAG-Lactones. DAG-lactones analogous to DAGs **17** and **18** were synthesized to contrast the binding of these two classes of ligands and to seek an increase in binding affinity from lactonization. A comparison of DAGs **19** and **20** with DAG-lactone **21** (Scheme 5), all of which have similar alkyl branches and log *P* values, revealed a 10-fold increase in binding affinity for the latter, which was explained on the basis of the entropic advantage of constraining the glycerol backbone.[25] DAG-lactones (*E*,*Z*-**22** and *E*,*Z*-**23**), conceptually derived by *sn*-2 lactonization of the less lipophilic DAG analogues **17** and **18**, were synthesized to study alkyl group predilection on a constrained glycerol backbone (Scheme 4). Interestingly, lactones *Z*-**22** and *E*-**22**, derived from the most potent DAG (**17**), did not show the expected 10-fold increase in binding. On the other hand, compound *Z*-**23**, derived from the weakest DAG **18**, showed a 33-fold increase in binding when compared to **18**, and even the less potent *E*-isomer exhibited a 10-fold increase. Since for the sake of synthetic expediency these DAG-lactones were synthesized as racemates, the potency for the active (*R*)-isomer should be approximately doubled. The above

Table 5. H-Bonding Interaction Energies (kcal/mol) and Buried Surface Area (Å²)of DAGs 17 and 18 at the C1 Domains of PK-Cα

	H-bonding		buried surface	
	C1a	C1b	C1a	C1b
		DAGs		
17 (*sn*-1)	−120.33	−115.30	547.59	563.74
17 (*sn*-2)	−61.22	−102.35	533.31	547.11
18 (*sn*-1)	−119.59	−107.60	548.92	555.47
18 (*sn*-2)	−87.52	−100.52	553.62	562.27

results led us to conclude that DAGs and DAG-lactones have opposite binding modes that can be reinforced or antagonized by the position of the branched alkyl chain (Figure 5C,D).

4.6. Molecular Modeling and the Question of *sn*-1 versus *sn*-2 Binding. Previous modeling studies were performed with the empty C1b domain of PK-Cδ directly obtained from the X-ray structure. However, since most of our biological data have been generated with the α-isozyme, homology models of the C1a and C1b domains of PK-Cα were built on the basis of the crystal structure of the C1b domain of PK-Cδ.[5] Since the above sets of DAGs (**17** and **18**) and DAG-lactones (*E*,*Z*-**22** and *E*,*Z*-**23**) provided the clearest evidence that PK-Cα can differentiate between *sn*-1 and *sn*-2 binding modes, the compounds were selected for docking studies with the PK-Cα isozyme using the program GOLD.[26,27] In addition to H-bonding energies, hydrophobic interactions were gauged by calculating the average difference in solvent-accessible surface area between the bound complex and the unbound ligand and receptor. The latter number is related to the nonpolar component of the solvation energy (i.e., the hydrophobic effect), and the size of the surface area that is buried upon ligand binding is a good measure of the strength of the hydrophobic interactions between ligand and receptor. Using the open DAGs **17** and **18** as examples (Table 5), one can see that H-bonding energy is stronger (lower values) with *sn*-1 binding. When the larger alkyl chain appears next to the *sn*-1 carbonyl, as in DAG **17**, the size of the buried surface area upon ligand binding is also larger with *sn*-1 binding, suggesting that this compound favors *sn*-1 binding (K_i = 40 nM, Scheme 4). However, when the larger alkyl chain is positioned next to the *sn*-2 carbonyl, as in DAG **18**, although the H-bonding energy is stronger with *sn*-1 binding, the size of the buried surface area is larger in the opposite *sn*-2 binding mode, resulting in poorer binding (K_i = 330 nM, Scheme 4).

The same contrasting forces were mirrored by the DAG-lactones **22** and **23** (data not shown).[27] In contrast to DAG, however, H-bonding for the lactones is stronger in the *sn*-2 orientation, a binding mode that could be reinforced by the increased size of the buried surface area when the bulkier side chain is next to the binding (*sn*-2) carbonyl as in *Z*-**23**. The goodness of this binding mode can be visually appreciated by the perfect fit of *Z*-**23** bound to the C1 domain of PK-Cα (Figure 7A). This type of analysis can also explain why the binding mode for a DAG-lactone with a branched α-alkylidene chain is sensitive to the stereochemistry of the double bond as the presence of the α,β-unsaturated system creates restricted rotation near the strictly conserved Gly253 binding site. This restricted rotation leads to a less than optimal fit for the branched chain of *E*-**23** (Figure 7B).

5. Structural Factors Influencing Isozyme-Specific PK-C−Membrane Interactions

The particular membranes of the target cell add a further dimension to the diversity provided by variation in the binding clefts of the various C1 domains. In vitro, PK-Cα and RasGRP, because of their different motifs for membrane interaction, have different dependence on the percent of phosphatidylserine in the phospholipid to support binding. The ability of these different DAG targets to discriminate between ligands could be shown to depend on the composition of the phospholipids in which they were assayed. This situation carries over into the intact cell, although there it is less clear whether the membrane composition is the only relevant variable.

Among C1 domains, structural differences outside of the binding cleft and other features of the PK-C isoforms within which the C1 domains are imbedded contribute to nonequivalence. In elegant studies, different C1 domains were shown to insert to different extents into artificial lipid monolayers.[28] Likewise, studies with mutations in the individual C1 domains within PK-C isoforms suggest nonequivalent roles for the first and second C1 domains, depending somewhat on the specific ligands.[29] Studies with synthetic C1 domains suggest this same conclusion, although the different approaches differ somewhat in the relative roles of different domains.[30]

5.1. Intracellular Localization and Isozyme Specificity. The specific nature of the hydrophobic interactions has great influence on the biological outcome. For a series of symmetrically substituted phorbol 12,13-diesters, we showed how the hydrophobicity of the side chains markedly influenced the kinetics and pattern of translocation of PK-Cδ within the cell.[31] Likewise, the sole difference between the potent tumor promoting derivative 12-deoxyphorbol-13-tetradecanoate and the antitumor promoting 12-deoxyphorbol-13-phenylacetate was the nature of the side chain. While the former induced rapid plasma membrane translocation and slower nuclear membrane translocation of a PK-Cδ-green fluorescent protein (GFP-fusion) construct, the latter induced the formation of intracellular patches and less defined but prominent nuclear membrane localization.[32] The DAG-lactone **21** induced yet a different pattern of translocation to the nuclear membrane and to a perinuclear region colocalizing with the Golgi.[32] This and other DAG-lactones also translocated β2-chimaerin, a protein with C1 domains responsive to DAG, to a similar perinuclear region and showed binding K_i ratios (PK-Cα/β2-chimaerin) as high as 6.[33] Such clear binding preference for β2-chimaerin in the case of **21** even surpassed PDBU [compound **21** (K_i = 0.9 nM); PDBU, K_i = 1.5 nM]. A more dramatic effect in terms of specific membrane translocation and isoform dependency was observed for the *Z*-isomer of DAG-

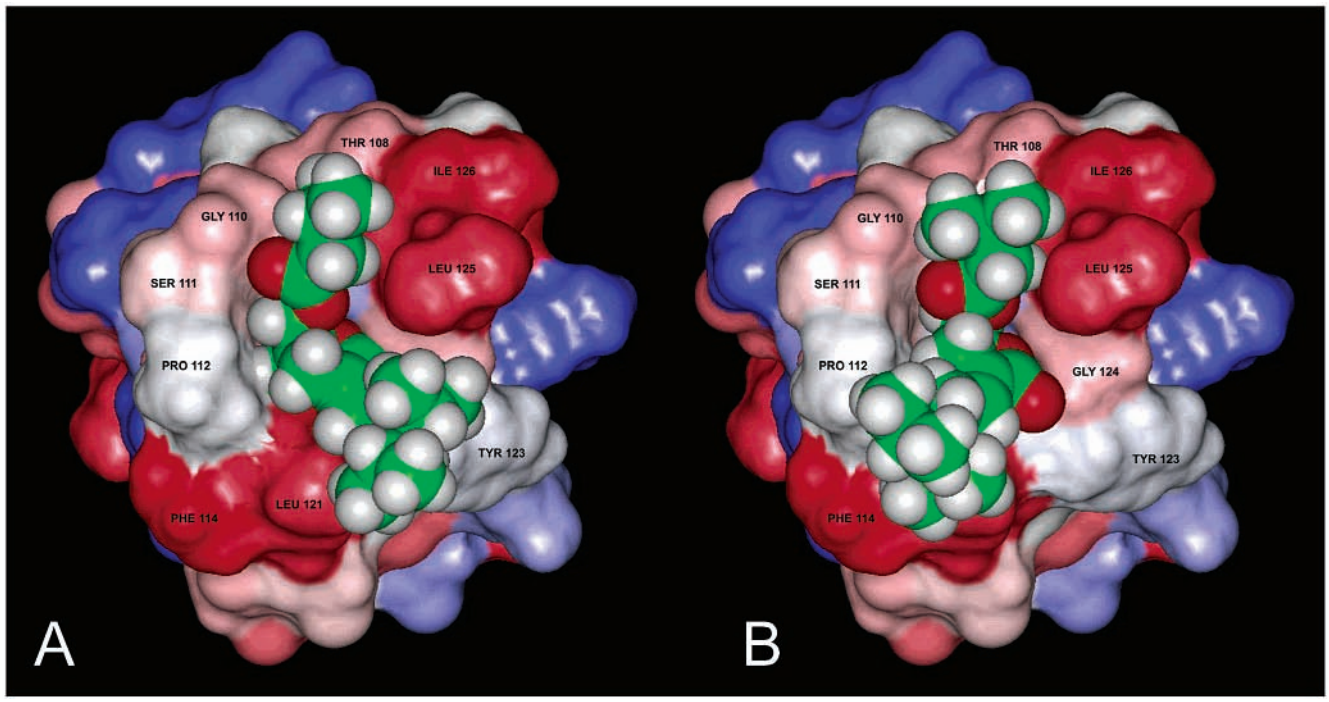


FIGURE 7. Space-filling models showing DAG-lactones *Z*-**23** (A) and *E*-**33** (B) bound to the C1b domain of PK-Cα.

lactone **24**, which in marked contrast to phorbol-12-myristate-13-acetate (PMA) caused the selective translocation of PK-Cα exclusively to the plasma membrane, whereas PK-Cδ was predominantly translocated to the nuclear membrane.[34] These results, which were incorrectly attributed to the *N*-hydroxyl amide isostere,[14,15] correspond in fact to the ester **24** (Table 6).

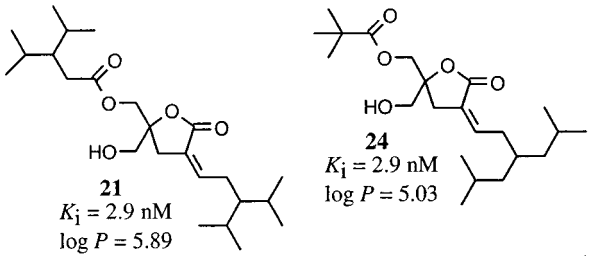
6. Antitumor Activity

The role of intracellular localization in determining functional activity is critical, and the ability of PK-Cs to phosphorylate their substrates depends not only on their intrinsic level of catalytic activity but also on their proximity to their potential substrates. Typically, PK-Cs translocate to different cellular compartments in response to ligand binding to the C1 domains. We surmise that differences in in vitro antiproliferative activity between DAG-lactones **21** and **24**—compounds with identical K_i values and similar lipophilicity—are most likely due to different patterns of subcellular translocation (Table 6).

7. Conclusions

The patterns that differentiate the mode of binding between DAGs and their corresponding lactones can now be explained. If the branched alkyl chain is adjacent to the *sn*-1 carbonyl, the DAG-lactone binds such as a DAG (*sn*-1) with little entropic benefit derived from lactonization (compare K_i values for **17** and *Z*-**22** and *E*-**22**, Scheme 4). Also, the *sn*-1 binding mode is insensitive to the *E*- and *Z*-stereochemistry of the double bond (compare K_i values for *Z*-**22** and *Z*-**22**). However, if the location of the branched alkyl chain is adjacent to the lactone carbonyl

Table 6. Comparative Testing in the NCI in Vitro Screen



	GI_{50}	GI_{50}
full panel MG-MID[a]	3.2×10^{-6} M	3.0×10^{-6} M
delta[b]	2.51	1.68
leukemia K-562	2.5×10^{-8} M	5.2×10^{-7} M
leukemia CCRF-CEM	1.0×10^{-5} M	7.9×10^{-8} M
nonsmall Cell (NCI-H322M)	1.1×10^{-5} M	6.3×10^{-8} M
colon cancer (COLO 205)	2.1×10^{-8} M	1.1×10^{-7} M
melanoma (SK-MEL-5)	1.0×10^{-5} M	8.7×10^{-8} M
breast HS-578T	2.6×10^{-8} M	7.7×10^{-7} M

[a] MG-MID is the calculated mean panel GI_{50} concentrations (M). GI_{50} is an interpolated value representing the concentration (M) at which percentage growth is inhibited 50%. [b] Delta is the number of log units by which the delta of the most sensitive line(s) of the panel differ from the corresponding MG-MID. The individual deltas are calculated by subtracting each log GI_{50} from the panel mean.

engaged in binding (i.e., *sn*-2), the binding affinity increases at least ca. 10-fold (compare K_i values for **18** and *Z*-**23** and *E*-**23**, Scheme 4). Furthermore, as expected for a branched α-alkylidene chain, such a binding mode is sensitive to the stereochemistry of the double bond. These studies have confirmed that specific contacts between the branched alkyl and the hydrophobic amino acids on the protein are indeed responsible for the observed discrimination in binding, as interactions with the lipid would be

expected to be less sensitive to the stereochemical disposition of the alkyl chains. Depending on the specific patterns of hydrophobic substitution on the ligand, these substituents are expected to make a further hydrophobic contribution with the cellular membrane. The combination of these two factors, under conditions of an appropriate lipid surface and other co-regulators, drives conformational change and membrane binding of the C1 domain. Our ability to dissect these binding forces with sets of structurally simple, yet highly potent DAG and DAG-lactones, represents a powerful tool to achieve isozyme specificity through molecular diversity.

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