Iridals Are a Novel Class of Ligands for Phorbol Ester Receptors with Modest Selectivity for the RasGRP Receptor Subfamily†

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Since 1990, the National Cancer Institute has performed extensive in vitro screening of compounds for anticancer activity. To date, more than 70 000 compounds have been screened for their antiproliferation activities against a panel of 60 human cancer cell lines. We probed this database to identify novel structural classes with a pattern of biological activity on these cell lines similar to that of the phorbol esters. The iridals form such a structural class. Using the program Autodock, we show that the iridals dock to the same position on the C1b domain of protein kinase C δ as do the phorbol esters, with the primary hydroxyl group of the iridal at the C3 position forming two hydrogen bonds with the amide group of Thr12 and with the carbonyl group of Leu 21 and the aldehyde oxygen of the iridal forming a hydrogen bond with the amide group of Gly23. Biological analysis of two iridals, NSC 631939 and NSC 631941, revealed that they bound to protein kinase C α with K_i values of 75.6 ± 1.3 and 83.6 ± 1.5 nM, respectively. Protein kinase C is now recognized to represent only one of five families of proteins with C1 domains capable of high-affinity binding of diacylglycerol and the phorbol esters. NSC 631939 and NSC 631941 bound to RasGRP3, a phorbol ester receptor that directly links diacylglycerol/phorbol ester signaling with Ras activation, with K_i values of 15.5 ± 2.3 and 41.7 ± 6.5 nM, respectively. Relative to phorbol 12,13-dibutyrate, they showed 15- and 6-fold selectivity for RasGRP3. Both compounds caused translocation of green fluorescent protein tagged RasGRP3 expressed in HEK293 cells, and both compounds induced phosphorylation of ERK1/2, a downstream indicator of Ras activation, in a RasGRP3-dependent fashion. We conclude that the iridals represent a promising structural motif for design of ligands for phorbol ester receptor family members.

Introduction

The lipophilic second messenger *sn*-1,2-diacylglycerol (DAG), generated as one arm of the phosphoinositide turnover pathway as well as indirectly through phospholipase D activity, plays a central role in cellular signaling.[1] DAG binds with high affinity to C1 domains, a class of zinc finger structures first characterized in protein kinase C (PKC).[2] It is now recognized, however, that PKC is only one of five families of proteins that have appropriate C1 domains to act as diacylglycerol receptors.[3] The other receptor families are the PKD family,[4] a distinct class of serine/threonine kinases; the chimaerins,[5] inhibitors of p21Rac; the munc-13 family,[6] proteins involved in synaptic vesicle priming; and the RasGRP family,[7−9] guanyl nucleotide exchange factors for Ras and Rap1. Of these families, the RasGRP family is of particular interest because it provides a direct link between diacylglycerol signaling and Ras activation.[7,10] Major, complementary issues are understanding how signaling by diacylglycerol can selectively utilize a subset of these 19 receptors and, conversely, how pharmacological ligands can be developed for subsets of these receptors.

Natural products have provided powerful tools for understanding the biological role and pharmacology of PKC and these other DAG receptor families. Indeed, the demonstration that the phorbol ester tumor promoters possessed specific cellular receptors, the identification of these receptors as PKC, and the recognition that the phorbol esters function as ultrapotent diacylglycerol analogues have been central to the elucidation of diacylglycerol as a signaling molecule. We now know that nature has devised multiple, structurally diverse, high-affinity solutions for diacylglycerol mimetics that bind to PKC and related receptors. These include the phorbol esters (diterpenes),[11] the bryostatins (macrocyclic lactones),[12,13] the teleocidins (indole alkaloids),[14] and

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† Abbreviations: DAG, *sn*-1,2-diacylglycerol; PKC, protein kinase C; rmsd, root-mean-square deviation; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate-13-acetate; MBP, maltose binding protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; MEK, ERK kinase.

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the aplysiatoxins (polyacetates).[15] These natural products have provided lead structures for medicinal chemistry and have revealed the opportunities for diverse biology arising from this chemical diversity. Thus, 12-deoxyphorbol 13-phenylacetate is a potent antitumor promoter,[16] in stark contrast to phorbol 12-myristate 13-acetate, the paradigm for tumor-promoting agents.[11] Likewise, bryostatin 1 is a functional antagonist for many PKC-mediated responses and, as of July 2001, is in 18 phase 1 or phase 2 clinical trials for a range of advanced cancers including esophageal, breast, ovarian, cervical, and prostate.[12,17]

The structures of several C1 domains have been determined by NMR, and the structure of the binary complex of phorbol 13-acetate with the C1b domain of PKC δ has been solved by X-ray crystallography.[18] These insights have been substantially extended by computer modeling to explore the structural basis of binding by the diverse classes of ligands for PKC.[19,20] The understanding that has emerged is as follows. Ligands insert into a hydrophilic cleft at the top of the C1 domain. The surface surrounding this cleft is hydrophobic, and the insertion of the ligand into the cleft, which positions hydrophobic residues of the ligand above the cleft, completes the hydrophobic surface and thereby promotes C1 interaction with the membrane. Different ligands do not possess a common set of pharmacophoric groups. Rather, the interaction of different ligands with the C1 domain reflects a combination of common and unique hydrogen bonds formed with the backbone of residues of the C1 domain.[19,20] Unfortunately, our understanding of ligand binding is still clouded because in vivo the C1 domain represents only a half-site, with the lipid membrane completing the complex. Modeling of the ternary C1/lipid/ligand complex represents an ongoing challenge.

The United States National Cancer Institute (NCI) conducts an anticancer drug discovery program in which approximately 10 000 compounds are screened every year in vitro against a panel of 60 human cancer cell lines from different organs.[21−25] Approximately 70 000 compounds have been tested since 1990, of which approximately 35 000 are "open" (nonconfidential) compounds. A number of studies have shown that although growth inhibitory activity for a single cell line is not informative, the activity patterns across the 60 cell lines provide incisive information on the mechanism of action of screened compounds and also on molecular targets and modulators within the cancer cells. Several algorithms have been introduced to use the activity information for discovery of anticancer drugs and for understanding of the molecular pharmacology of cancer. The COMPARE algorithm has proven to be very useful for finding agents with activity patterns similar to that of a "seed" compound and for finding compounds with activity patterns that correlate well across the 60 cell lines with the expression levels of particular molecular targets.[26−29] Weinstein's group at the NCI has developed an "information-intensive" approach to use this anticancer database for studies of molecular pharmacology of cancer and for anticancer drug discovery.[30−33]

A number of ligands for PKC have been evaluated in the NCI screening program. In the present study, the iridals[34] were identified as ligands acting through the diacylglycerol signaling pathway on the basis of a profile of cell line responsiveness similar to that of known PKC ligands. Indeed, one member of this class has previously been shown to act as a ligand for PKC.[35] Computer modeling confirmed that the iridals can dock to the C1 domain and do so in a fashion consistent with other high-affinity ligands. Finally, we evaluated two structural subclasses of the iridals as ligands for the RasGRP family of diacylglycerol/phorbol ester receptors, analyzing binding, translocation, and induction of downstream signaling. We conclude that the iridals represent a promising lead structure for design of novel ligands targeted to the diacylglycerol/phorbol ester receptors. Moreover, the iridals are the first example of ligands at least modestly selective for a RasGRP family member rather than for PKC.

Experimental Section

Materials. Iridals NSC 631939 ((−)-(6*R*,10*S*,11*S*,18*R*,22*S*)-26-hydroxy-22-α-methylcycloirid-16-enal) (iripallidal) and NSC 631941 ((+)-(6*R*,10*S*,11*S*,14*S*,26*R*)-29-acetoxy-26-hydroxy-15,-28,16,17-tetradehydro-14,15-dihydrospiroiridal) were isolated as described previously.[34] Compounds were stored at −20 °C because of lability, especially of the latter compound.

Correlation Analysis of the NCI Anticancer Database To Identify Potential PKC Ligands. To facilitate our extensive data-mining efforts of the NCI anticancer drug database, we created our own version of the COMPARE program, which produced essentially the same results as the NCI version of the COMPARE program.[26] Of the 35 000 "open" compounds (nonconfidential compounds) screened by the NCI since 1990, 6366 compounds were found to have a GI_{50} value less than 1 μM against at least one cancer cell line, where GI_{50} is the concentration required to inhibit cell growth by 50% compared to untreated control. These 6366 compounds were considered to be of special interest because of their fairly potent antiproliferation activity against cancer cells. We reasoned that these potent compounds are likely to bind specific molecular targets with high affinity. A local database, which contained these 6366 compounds and their GI_{50} values for each cell line, was created and used in the analysis reported in this study. Standard Pearson's correlation coefficient (r) was calculated based on the following equation

$$r = \frac{\sum_i^N (x_i - x)(y_i - y)}{\sqrt{\sum_i^N (x_i - \bar{x})^2}\sqrt{\sum_i^N (y_i - \bar{y})^2}} \quad (1)$$

where x and y represent values for two different compounds, x_i and y_i represent the logarithm of $1/GI_{50}$ ($\log(1/GI_{50})$) on a single cell line (i) for x and y, respectively, $\bar{x}$ and $\bar{y}$ are the average values in all 60 cell lines for x and y, respectively, and N is the total number of cell lines tested for x and y. The range of r is from −1 to 1, with the higher value indicating greater similarity.

Computational Docking Studies. The Autodock program (version 3.0) was employed to perform the docking study for NSC 631939.[36] The crystal structure of PKC δ C1b in complex with phorbol 13-acetate was used as the starting structure for the protein.[18] The protein was treated using the united-atom approximation, and only the polar hydrogen atoms were added to the protein. Kollman united-atom partial charges were assigned. All crystallographic water molecules were removed. Atomic solvation parameters and fragmental volumes were assigned to the protein atoms using the AutoDock utility AddSol. The initial 3D structure of the ligand was built using the molecular modeling program SYBYL (SYBYL is provided by Tripos Associates, Inc., 1699 South Hanley Road, Suite 303,

St. Louis, MO 63144-2913) The ligand was treated in SYBYL initially as all atom entities; i.e., all hydrogens were added. Partial atomic charges were calculated using the Gasteiger−Marsili method.

During the docking process, the overall orientation of the ligand was unrestrained. The rotatable bonds in the molecule were defined using an AutoDock utility, AutoTors, which also unites the nonpolar hydrogens added by Sybyl for the partial atomic charge calculation. The grid maps were calculated using AutoGrid. We used grid maps with $60 \times 60 \times 60$ points and a grid-point spacing of 0.30 Å. The centers of the grid maps were defined using the coordinates of the center of mass of phorbol 13-acetate in the crystal structure. A total of 100 docking simulations were performed. The step sizes used in docking simulation were 0.2 Å for translation and 5° for orientation and torsion. In the analysis of the docked structures, the clustering tolerance was set to 1.0 Å for the root-mean-square deviation (rmsd) for the ligand. The LGA docking method was employed. The maximum number of energy evaluations was set as 1.5×10^6.

Binding Assay. Affinities of the iridals for PKC α or RasGRP3 were determined by competition of [^{3}H]phorbol 12,-13-dibutyrate ([^{3}H]PDBu) binding. PKC α was partially purified as described elsewhere.[37] The coding sequence of human RasGRP3 (KIAA0846) was subcloned into the vector pMAL-c2 (New England Biolabs, Beverly, MA) downstream from the maltose-binding protein (MBP) gene. The construct, which encoded the fusion protein MBP/RasGRP3, was expressed in bacteria and partially purified using amylose beads (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. [^{3}H]PDBu binding to PKC α or RasGRP3 was measured using the poly(ethylene glycol) precipitation method developed in our laboratory.[38] The iridals were dissolved in dimethyl sulfoxide (Pierce, Rockford, IL) and diluted in 50 mM Tris-Cl, pH 7.4, containing 1 mg/mL γ-globulins (Cohn Fraction ll, lll, Sigma, St. Louis, MO) prior to the binding assay. Incubations with radioligand (3.0 nM [^{3}H]PDBu, specific activity of 21 Ci/mmol, New England Nuclear, Boston, MA) were performed in 50 mM Tris-Cl, pH 7.4, containing 2 mg/mL γ-globulins, 0.1 mM Ca^{2+}, and 0.1 mg/mL phosphatidylserine (Avanti Polar Lipids, Alabaster, AL). Nonspecific binding was determined in the presence of 30 μM phorbol 12,-13-dibutyrate (LC Laboratories, Woburn, MA). The tubes were incubated for 5 min at 37 °C for PKC α and for 5 min at 18 °C for RasGRP3. The assay was terminated by incubation at 0 °C for 5 min, followed by protein precipitation with 200 μL of 35% poly(ethylene glycol) 6000 (EM Science, Gibbstown, NJ). The amounts of radioactivity associated with the pellet (bound) and the supernatant (free) were determined by scintillation counting.

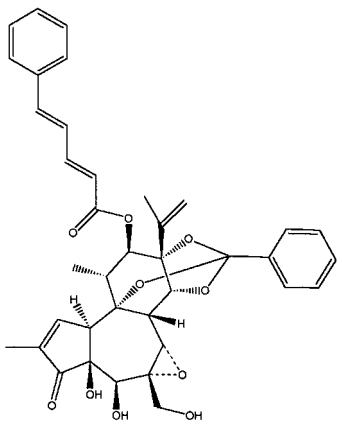
Cell Culture. HEK-293 cells were cultured in Eagle's Minimum Essential Medium adjusted to contain 0.1 mM nonessential amino acids (ATCC, Manassas, VA) and supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Gaithersburg, MD). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Expression of RasGRP3/GFP Fusion Protein. The cDNA of RasGRP3 was amplified by a polymerase chain reaction and subcloned into the pQBI25 vector (Quantum Biotechnologies, Inc., Montreal, Canada) using the Nhe1 site; this generated a fusion protein (RasGRP3/GFP) between the C terminus of RasGRP3 and the N terminus of the green fluorescent protein (GFP) tag.[39] HEK-293 cells were grown on 40 mm round coverslips (Bioptechs, Inc., Butler, PA) at a seeding density of 2.5×10^5 cells per dish. Transient transfection was conducted using LipofectAMINE PLUS (Life Technologies, Inc., Rockville, MD) according to the manufacturer's protocol. The fluorescence became detectable 24 h after transfection, and all experiments were performed 48−72 h after transfection. Ligands for RasGRP3 were dissolved in dimethyl sulfoxide and diluted to the indicated concentrations in DMEM without phenol red and were supplemented with 10% fetal bovine serum (Life Technologies, Inc., Rockville, MD). The final concentration of DMSO was 0.1%. Confocal fluorescent images were collected with a Bio-Rad MRC 1024 confocal scan head (Bio-Rad, Hercules, CA) mounted on a Nikon Optiphot microscope with a 60× planapochromat lens. Excitation at 488 nm was provided by a krypton/argon gas laser with a 522/532 nm emission filter for green fluorescence. For living cell imaging, a Bioptechs Focht Chamber System (Bioptechs, Butler, PA) was inverted and attached to the microscope stage with a custom stage adapter. The cells plated on the 40 mm coverslip were enclosed in the chamber and connected to a temperature controller set at 37 °C, and media was perfused through the chamber with a Lambda microperfusion pump (Instech Laboratories, Inc., Plymouth Meeting, PA). Each compound was delivered to the cells by a syringe pump of 2.5 mL capacity (Yale Apparatus, Wantagh, NY). Sequential images were collected for 30 min using LaserSharp software.

ERK Activation Assays. HEK-293 cells were grown in 60 mm dishes and serum-starved overnight before treatment with different concentrations of the iridals or vehicle for 15 min at 37 °C. When indicated, a pretreatment with the PKC inhibitor GF-109203X (5 μM) was performed 30 min before the addition of the iridal. Cells were washed twice in 1X Dulbecco's PBS and harvested in 60 μL of lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and protease inhibitors). After an 8 s sonication pulse, a sample of 50 μg of total protein was prepared in 2X Laemmli buffer and boiled for 5 min. Samples were subjected to SDS-PAGE using 4−20% precast gels (Invitrogen, Carlsbad, CA) followed by transfer onto nitrocellulose membranes. The membranes were blocked with 1X PBS containing 5% milk (PBS milk) at room temperature for 20 min and then incubated overnight at 4 °C with PBS milk containing 2 μg/mL phospho-ERK1/ERK2 antibody (New England Bio Labs, Beverly, MA). After being washed for 20 min with 1X PBS containing 0.05% Tween-20 (PBS-Tween), membranes were incubated with horseradish peroxidase conjugated antirabbit antibody (Bio-Rad, Hercules, CA) for 1 h at room temperature (1/2000 dilution in PBS milk). After 1 h of being washed in PBS-Tween, resolved proteins were detected using SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL). For subsequent probing with an antibody against total ERK1/ERK2 (New England Biolabs, Beverly, MA) membranes were reused after stripping the phospho-ERK1/ERK2 antibody with the Western Blot Recycling Kit (Alpha Diagnostic Intl., San Antonio, TX).

Results

Identification of Compounds in the NCI Anticancer Database, Showing a Pattern of Biological Activity Highly Correlated with That of Mezerein. We probed the NCI 60 human cancer cell line database to identify compounds whose pattern of antiproliferative activities correlated with that of mezerein. Although



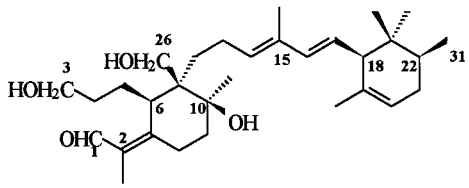
Mezerein (**NSC 239072**)

Table 1. Correlation Analysis of the Patterns of Antiproliferation Activities for 35 000 NCI Compounds against the NCI 60 Human Cancer Cell Lines with That of Mezerein

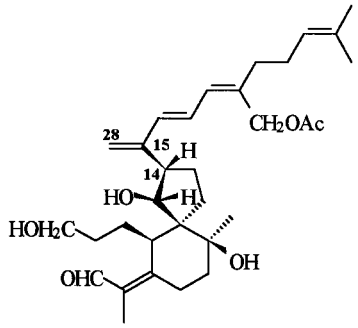
rank	NSC no.	chemical name (or chemical class)	Pearson's correlation coefficient (*r*)	range of activities in inhibition of cell growth in the NCI 60 cell lines ($\log(1/GI_{50})$)
1	239 072	mezerein	1.000	5.8−9.3
2	266 186	huratoxin	0.822	4.7−8.0
3	654 239	cytoblastin	0.802	4.0−6.2
4	623 310	prostratin (phorbol)	0.753	3.0−6.5
5	339 875	phorbol ester	0.677	4.0−6.0
6	688 228		0.627	4.0−6.0
7	252 940	gnidimacrin	0.615	4.0−8.0
8	627 960		0.571	3.2−7.2
9	688 220		0.571	4.7−6.3
10	631 939	iridal analog	0.565	4.6−8.0
11	688 222	phorbol ester	0.558	5.1−6.9
12	688 235		0.555	5.4−7.9
13	645 597		0.543	4.0−6.1
14	629 156		0.524	4.0−6.2
15	329 507	phorbol ester	0.520	4.3−7.4
16	688 239		0.518	4.6−6.0
17	686 038		0.505	4.0−6.5
18	631 941	iridal analog	0.505	4.4−8.0

phorbol 12,13-dibutyrate (PDBu) or phorbol 12-myristate-13-acetate (PMA) might have been preferable as the "seed" compound in such an analysis, given their extensive use as PKC probes, neither PDBu nor PMA has been tested in the NCI screen. Mezerein binds to PKC with a subnanomolar affinity and potently inhibits cell growth in the NCI panel of 60 human cancer cell lines.

Of the 35 000 "open" compounds (nonconfidential compounds) screened by the NCI since 1990, 6366 compounds were found to have a GI_{50} value less than 1 μM against at least one cancer cell line. The correlation analysis was thus performed on these 6366 "potent" compounds. Out of these compounds, there are only 18 compounds (Table 1) whose patterns of antiproliferation activities have a Pearson's correlation coefficient (*r*) greater than 0.5 compared with that of mezerein. As expected, the pattern of activity of mezerein correlates with itself with an *r* value of 1. The other top four compounds in Table 1 are confirmed PKC ligands. Huratoxin (NSC 266186) is a potent PKC ligand that, like mezerein, falls into the daphnane subclass of phorbol ester related diterpenes.[40] Cytoblastin (NSC 654239) is an analogue of teleocidin and indolactam (ILV), a structurally distinct class of potent PKC ligands.[41] Prostratin (NSC 623310) is a phorbol ester analogue, which has good affinity for PKC but, rather than being a tumor promoter, displays potent antitumor-promoting activity.[42] Diacetyl-12-octadienoyl-4-deoxy phorbol (NSC 339875) is another phorbol ester. Interestingly, the activity patterns of two other phorbol esters (NSC 688222 and 329507) only have moderate correlation with that of mezerein, viz. 0.558 and 0.520, respectively. This suggests that a correlation coefficient of 0.5 between the patterns of activity of two ligands may still indicate the same molecular mechanism of action. Gnidimacrin (NSC 252940) is a confirmed PKC ligand and has a chemical structure similar to that of mezerein.[43] Of the other compounds whose activity patterns correlate well with that of mezerein, two (NSC 627960 and 645597) have IC_{50} values above 10 μM in all but one of the cell lines tested, whereas eight (NSC 688228, 688220, 631939, 688235, 629156, 688239, 686038, and 631941) have greater potency. These latter eight compounds are thus interesting potential ligands that may either bind to PKC directly or target the PKC-mediated signaling pathway. Unfortunately, six such compounds ((NSC 688228, 688220, 688235, 629156, 688239, and 686038) are no longer available in the NCI repository, so their binding to PKC could not be measured experimentally. The two remaining compounds, NSC 631939 and NSC 631941, are structurally related iridals.[44,45]



NSC 631939



NSC 631941

The iridals are unusual triterpenoids originally isolated from rhizomes and roots of various *Iris* species. These plants have long been known for their medicinal properties (Figure 1). Compared to other classes of PKC ligands, such as the phorbol esters, mezerein, and bryostatin, the iridals have relatively simple chemical structures. In the NCI 60 cell line screen, both NSC 631939 and NSC 631941 display quite potent activity in inhibition of cell growth, with GI50 values ranging between micromolar and nanomolar concentrations.

Computational Docking Studies of NSC 631939 to PKC δ C1b Domain. The correlation analysis showed that NSC 631939 and NSC 631941 are potential PKC ligands. We performed computational docking studies of NSC 631939 to investigate whether it could bind to the C1 domain of PKC in a fashion similar to that of mezerein and the phorbol esters. The chemical structure of NSC 631939 resembles those of PDBu and PMA in having a hydrophobic group and a hydrophilic ring system. Most high-affinity PKC ligands have one or more lengthy hydrophobic groups. X-ray crystallographic analysis of the binding of phorbol 13-acetate to PKC δ C1b and molecular modeling studies of several PKC ligands to PKC have both shown that the lengthy hydrophobic group(s) is (are) primarily responsible for the interactions between ligands and lipid. Thus, for

Figure 1. Illustration of *Iris pallida,* taken from an 1887 compendium of medicinal plants.[62]

simplicity the long hydrophobic group was shortened in our docking studies of the binding of NSC 631939 to PKC.

Computational docking using the Autodock program led to a highly converged binding model for this ligand. Out of a total of 100 independent docking simulations, 13 of them converged to a cluster of virtually identical binding models with the lowest predicted binding free energy by the Autodock program. The root-mean-square deviation value for the ligand between the models in this cluster is less than 1 Å. Furthermore, 16 and 29 additional simulations converged to the next two binding models with the second and third lowest predicted binding free energies. Superposition of these three top binding models revealed that they are very similar. Therefore, out of a total of 100 independent docking simulations for NSC 631939, 58 of them essentially converged to a single binding model for this ligand.

Our docking results showed that NSC 631939 binds to the PKC δ C1b domain in a highly complementary manner, forming a number of optimal hydrogen bonds with the protein (Figure 2). The primary hydroxyl group at the C3 position forms two hydrogen bonds with the backbone amide group of Thr12 and the backbone carbonyl group of Leu21, thus mimicking the primary hydroxyl group at the C20 position in phorbol esters as revealed by the X-ray structure. The aldehyde group at the C2 position in NSC 631939 forms a strong hydrogen bond with the backbone amide group of Gly23, thus mimicking the carbonyl group at the C3 position in the phorbol esters. In the X-ray structure, the hydroxyl group at the C9 position in phorbol 13-acetate does not

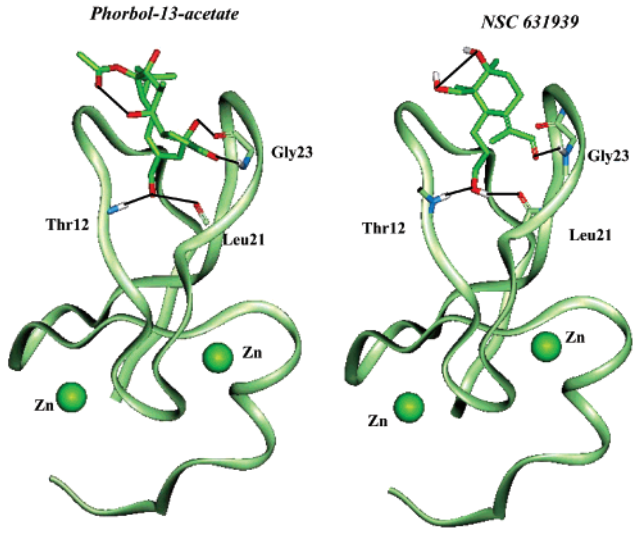


Figure 2. (a) Interaction between phorbol-13-acetate and PKC δ C1b domain as obtained by an X-ray crystallographic study.[18] Four crucial hydrogen bonds formed between phorbol 13-acetate and the protein and one intramolecular hydrogen bond in phorbol-13-acetate are depicted by black lines. (b) Interaction between an iridal analogue (NSC 631939) and PKC δ C1b domain as obtained by computational docking simulations. Three crucial hydrogen bonds formed between NSC 631939 and the protein and one intramolecular hydrogen bond in NSC 631939 are depicted by black lines. Protein structure is shown with ribbon representation, and the ligands are shown in liquorice.

have a specific hydrogen bond interaction with PKC but forms an intramolecular hydrogen bond with the carbonyl group of the ester at the C13 position. Interestingly, the primary hydroxyl group at the C26 position in NSC 631939 closely mimics the hydroxyl group at the C9 position, while the hydroxyl group at the C10 position in NSC 631939 mimics the hydroxyl group at the C12 position in phorbol 13-acetate. Furthermore, these two hydroxyl groups in NSC 631939 form a strong intramolecular hydrogen bond, as observed for phorbol 13-acetate in the X-ray structure. The hydrogen bond formed between the hydroxyl group at the C4 position in phorbol 13-acetate and PKC is missing in the predicted binding model between NSC 631939 and PKC. However, this hydrogen bond is known to be of minimal importance because removing the hydroxyl group at the C4 position in the phorbol ester results in only a 2-fold reduction in binding affinity to PKC.[46] We conclude from our computational docking studies that NSC 631939 binds to the PKC C1b binding domain in a manner very similar to that of phorbol esters in terms of the overall interactions with PKC.

Direct Analysis of Binding of Iridals to PKC α and to RasGRP3. Two iridals, NSC631939 and NSC631941, were available in sufficient quantity for selected, direct analysis. Binding affinity to PKC α has provided a consistent standard for structure−activity comparisons. NSC631939 bound with a K_i of 75.6 ± 1.3 nM (n = 3); the K_i for NSC631941 was 83.6 ± 1.5 nM (n = 3) (Figure 3). For comparison, the K_d for PDBu was 0.3 nM.

The PKC family of isoforms represents only one of five families of proteins that contain C1 domains capable of high-affinity interaction with diacylglycerols and phorbol esters. Among the other families, the RasGRP family

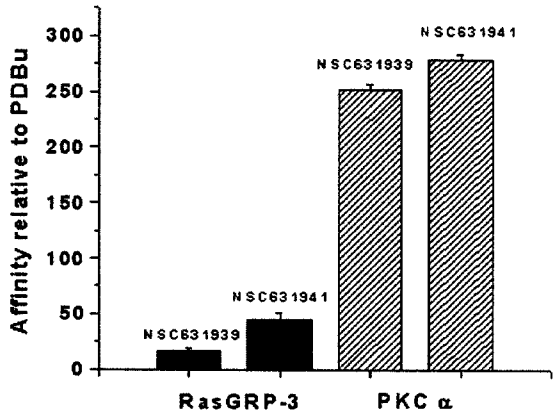


Figure 3. Comparison of binding of iridals to RasGRP3 and PKC α. The binding affinities of the iridals for RasGRP3 and PKCα were determined by inhibition of binding of [^{3}H]PDBu as described in Experimental Section and expressed relative to the affinities for PDBu. Values are the mean for three experiments; error bars are ±SEM.

is of particular interest because it provides a direct link between diacylglycerol/phorbol ester signaling and Ras activation. We have identified two family members so far, RasGRP and RasGRP3, which bind phorbol esters.[39,47] Of these, the tissue distribution of RasGRP is limited to brain, T-cells, and certain cells of the kidney,[7,9,10] whereas RasGRP3 shows a much broader tissue distribution (ref 9 and unpublished observations). We therefore examined the ability of the iridals to bind to RasGRP3. The K_i of NSC631939 was 15.5 ± 2.3 nM ($n = 3$); the K_i of NSC631941 was 41.7 ± 6.5 nM (Figure 3). Both compounds thus showed higher affinity for RasGRP3 than for PKC α. In contrast, RasGRP3 showed somewhat weaker affinity for PDBu ($K_d = 0.94 \pm 0.03$ nM) than did PKC α ($K_d = 0.30 \pm 0.05$ nM). NSC631939 and NSC631941 thus showed a relative selectivity for RasGRP3 compared to PDBu of 15- and 6-fold, respectively.

Induction of Translocation of RasGRP3 by Iridals. Our understanding of the signaling mechanism of C1 domains is that they function as hydrophobic switches.[18] Ligand binds to a hydrophilic cleft set within a hydrophobic surface. When the hydrophilic cleft is filled and covered with a hydrophobic cap, the ligand enhances the interaction of the C1 domain either with the lipid membrane or with other hydrophobic surfaces. This mechanism provides a biochemical rationale for the observation that the phorbol esters and related ligands induce translocation of PKC and related phorbol ester receptors. From the exploitation of fusion proteins between green fluorescent protein and phorbol ester receptors, it is now possible to visualize this translocation in intact cells in real time. We have previously described the translocation of RasGRP3/GFP from the cytoplasm to fibrillar structures and the nuclear membrane in response to phorbol 12-myristate-13-acetate or diacylglycerol.[39] The iridals NSC631939 and NSC631941 likewise induce a similar pattern of translocation, with clear changes in the pattern of localization within 5 min (Figure 4). Although the amount of compound available was not sufficient for detailed analysis of dose-response relations, the compounds were approximately 30-fold less potent than phorbol 12-myristate-13-acetate.

Induction of ERK1/2 Phosphorylation in Response to the Iridals. RasGRP3 functions as a GEF (guanyl nucleotide exchange factor) for the low molecular weight GTPases Ras and Rap1.[9] Ras activation in turn stimulates signaling through the Raf/MEK/ERK kinase pathway.[48] Independently, PKC signals through this pathway by activation of Raf.[49] We have described previously that phorbol esters activate RasGRP3 in intact cells, as evidenced by an enhanced level of phosphorylated ERK1/2 downstream in this pathway.[39] The iridals NSC631939 and NSC631941 likewise induce phosphorylation of ERK1/2 in a RasGRP3-dependent fashion (Figure 5). HEK293 cells were transfected with RasGRP3 and incubated in low serum to reduce the level of endogenous activity of the ERK1/2 pathway. Cells were then treated with increasing doses of NSC631939 or NSC631941 in the absence or presence of GF109203X, and the level of phosphorylated ERK1/2 was detected by Western blotting using antibodies specific for the phosphorylated form of ERK1/2. Because of the presence of endogenous PKC in the HEK293 cells, phosphorylation of ERK1/2 reflects the activity through both the PKC and RasGRP3 pathways. Inclusion of the PKC inhibitor GF109203X under these conditions fully blocks the PKC response,[39] permitting the contribution of RasGRP3 stimulation to be distinguished. Under these conditions, the iridals induced ERK1/2 phosphorylation in a dose-dependent fashion, with somewhat greater response observed for NSC631939, consistent with its greater potency.

Discussion

The modeling demonstrates that the iridals bind to the C1 domain in a fashion analogous to that previously described for the phorbol esters. The primary hydroxyl group at C3 corresponds to the primary hydroxyl group on the phorbol ester, whereas the aldehyde assumes a function similar to that of the C3 carbonyl group of the phorbol ester. The hydrophobic side chain presumably functions to provide membrane anchoring, in analogy to the ester substituents of the phorbol ester. Finally, although the remaining two hydroxyl groups do not interact with the C1 domain, it seems possible that they interact with the phospholipid headgroups in the ternary binding complex. In the case of constrained diacylglycerols, we have clearly demonstrated that the carbonyl group, which does not bind to the C1 domain, nevertheless makes a major contribution to binding affinity, presumably through phospholipid headgroup interactions.[50] Consistent with the previous structure−activity relations using piscicidal activity as an end point,[51] the spiro ring is not positioned to interact with the C1 domain and it does not enhance binding affinity. The core structure of the iridals thus provides one of the simplest lead structures for the design of novel ligands targeted to C1 domains.

The central role in signal transduction played by the diacylglycerol/phorbol ester pathways makes them attractive therapeutic targets. At the same time, their widespread involvement imposes substantial demands for selectivity for subsets of these pathways. Several strategies have been used. The first strategy has been inhibitors targeted to the kinase domain of the PKCs. LY333531, a selective inhibitor of PKC β enzymatic

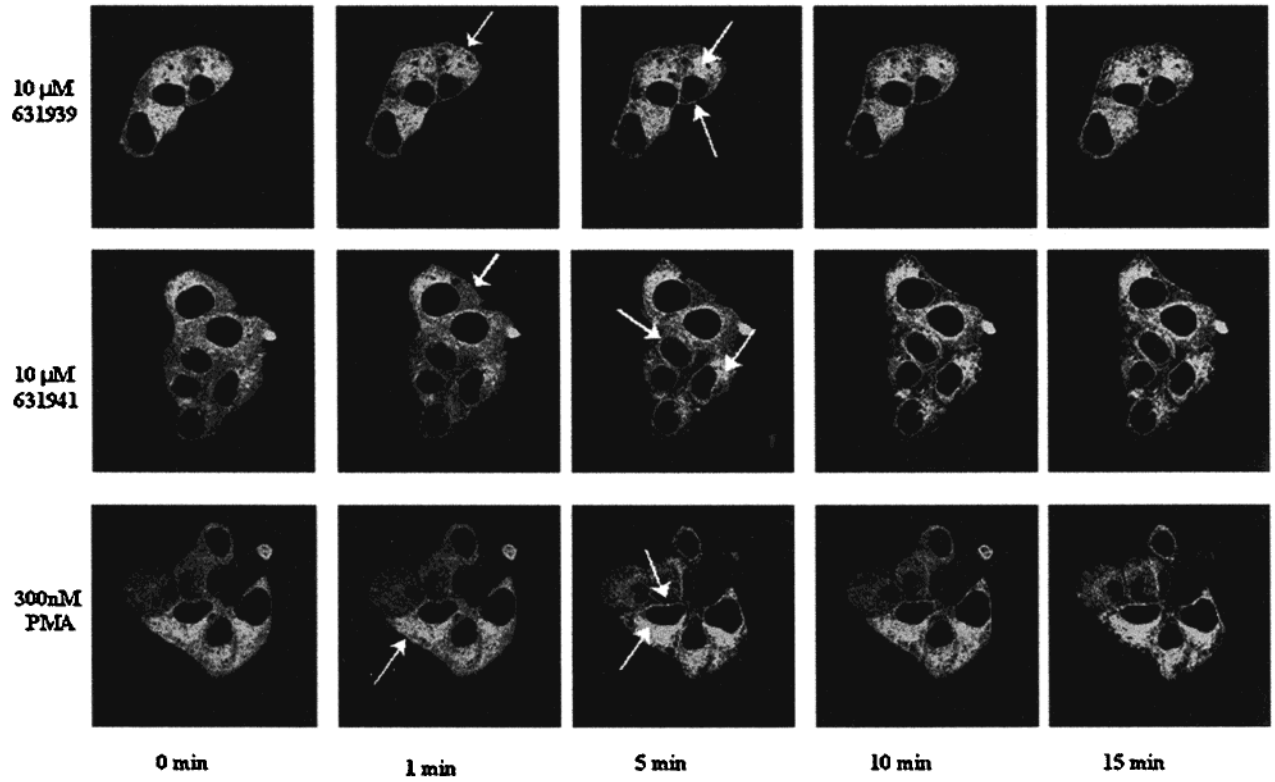


Figure 4. Translocation of RasGRP3/GFP by iridals and PMA. RasGRP3/GFP was expressed in HEK-293 cells. Cells were treated as indicated with iridals NSC631939 or NSC631941 or with PMA, and the localization of RasGRP3/GFP was determined by confocal microscopy as a function of time. Images shown are of cells before treatment or after treatment for 15 min as indicated. At least three experiments were performed, and images shown were representative. Arrows at 1 min indicate early and slight translocation of RasGRP3/GFP to the plasma membrane. Arrows at 5 min indicate major translocation of RasGRP3/GFP to the nuclear membrane and perinuclear region.

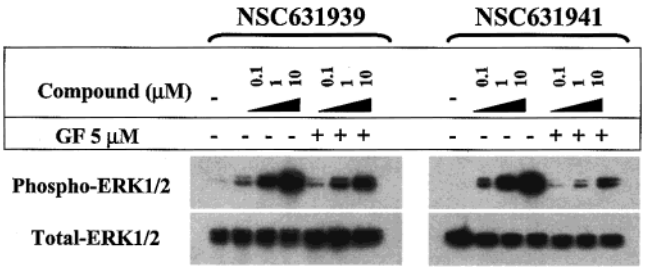


Figure 5. Activation of ERK by iridals. HEK-293 cells transfected with RasGRP3 were serum-starved overnight and then treated with NSC631939 or NSC631941 (0.1−10 μM) for 15 min at 37 °C. Where indicated, cells were pretreated for 30 min with a PKC inhibitor, GF109203X, at 5 μM final concentration. This treatment fully eliminated the phorbol ester mediated ERK phosphorylation in the control, vector-transfected cells. A total of 50 μg of protein from total cell lysates was separated by SDS-PAGE, and Western Blot was performed as indicated in Experimental Section. Activation of ERK was evaluated by measuring the level of phosphorylated ERK1/2 proteins (Phospho-ERK1/2) using specific anti-phospho ERK antibodies. The level of phosphorylated and non-phosphorylated ERK proteins (Total-ERK1/2) was also determined for comparison. Results shown are representative of three independent experiments.

activity, is currently in clinical trials for treatment of vascular complications of diabetes.[52,53] Rottlerin, an inhibitor with some selectivity for the PKC δ isoform, is widely used as a research tool.[54] The second strategy has exploited differences in the variable domains of the PKCs involved in intra- and intermolecular interactions. Peptides based on sequences in these interacting surfaces have proven to be valuable for manipulation of PKC ϵ in experimental situations.[55,56] The third approach, which can address the role of the non-PKC receptors as well as PKC in diacylglycerol/phorbol ester signaling, has been with compounds targeted to the C1 domains. Bryostatin 1, which is in clinical trials as a cancer chemotherapeutic agent, has a unique biphasic dose response for down regulation and protection of PKC δ.[57,58] Constrained diacylglycerol derivatives have been designed with nanomolar affinity and with modest 6-fold selectivity for β2-chimaerin relative to PKC α.[59] The iridals now provide the first example of compounds with modest (5-fold) selectivity for RasGRP3 relative to PKC α.

Although the conventional wisdom for the blocking of signaling pathways has been through enzyme inhibitors, as exemplified for PKC with LY333531, the diacylglycerol/phorbol ester signaling pathway has provided alternative paradigms as well. It is now clear that different members of these pathways may serve antagonistic functions. Thus, PKC δ is antiproliferative in NIH 3T3 cells whereas PKC ϵ stimulates proliferation.[60] An agonist for PKC δ may consequently achieve the same objective as an antagonist for PKC ϵ. A second approach is through inappropriate positional control. As characterized in detail for PKC δ, different ligands may cause translocation to different cellular compartments.[61] Since localization will determine access to substrates or binding partners, localization to the inappropriate compartment will prevent access to differently localized substrates and will produce apparent antagonism. Novel

structures based on the iridals should provide new opportunities for exploiting such strategies.

The biology of the iridals has been consistent with activity on the diacylglycerol/phorbol ester pathway. *Iris pallida* is a medicinal plant long known for its purgative properties, similar to croton oil, the source of the phorbol ester. Recently, the iridal derivative 28-deacetylbelamcandal was reported to induce differentiation of HL-60 promyelocytic leukemia cells, to release tumor necrosis factor α in mouse skin, and to cause mouse skin tumor promotion, all responses typical of the phorbol esters, although not unique to them.[35] The relative potencies for these three responses, compared to that of phorbol 12-myristate 13-acetate, were 170-, 500-, and 100-fold less potent, respectively. Finally, 28-deacetylbelamcandal was found to inhibit binding of [^{3}H]PDBu to PKC (a mixture of isoforms) with a 50% inhibitory concentration of 200 nM and to activate PKC enzymatic activity with an ED_{50} of 200 nM.[35] Our findings extend these results to another class of phorbol ester receptors, to a broader range of structures, and to further end points of response. They further reveal the nature of the interaction of the iridals with the C1 domain of PKC.

The central importance of diacylglycerol signaling in cellular functioning is highlighted by the impressive array of high-affinity ligands, which nature has devised presumably for chemical defensive purposes. The iridals afford us with yet another opportunity to build on this diversity provided by nature in the development of novel therapeutics.

Conclusions

By probing the U.S. National Cancer Institute in Vitro Anticancer Drug Discovery Screen database with a variant of the program COMPARE, we identified two compounds, structurally related iridals, with a pattern of biological activity similar to that of the phorbol esters. Using the program Autodock, we showed that the iridals dock to the same position on the C1b domain of protein kinase C δ as do the phorbol esters, with the primary hydroxyl group of the iridal at the C3 position forming two hydrogen bonds with the amide group of Thr12 and with the carbonyl group of Leu 21 and the aldehyde oxygen of the iridal forming a hydrogen bond with the amide group of Gly23. Biochemical characterization demonstrated that the iridals bind not only to PKC but also to the novel phorbol ester receptor and Ras activator RasGRP3, showing modest selectivity for RasGRP3. In intact cells, the iridals induce RasGRP3 translocation and downstream signaling through the ERK pathway. The relatively simple structures of the iridals compared to those of phorbol esters and their modest selectivity for RasGRP3 make these compounds a promising structural motif for the design of ligands for phorbol ester receptor family members.

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