Original Research Article

Mechanisms of Phorbol Ester Toxicity, Determined by Molecular Modelling

Abstract

Phorbol esters are toxic phytochemicals, whose main biological target is protein kinase C. They bind irreversibly to the protein, causing cell damage. Using computer modelling, we have determined, for the first time, features and mechanisms that lead to the toxicity of phorbol esters.

Protein kinase C – delta (PKC-δ) was used as a target protein in computational docking studies with phorbol esters that differ in molecular structure. Binding conformations and stability of ester linkages were analyzed to evaluate their relationship with experimental observations. Results show that an active phorbol ester must exhibit two features: interaction with specific amino acid residues at the binding site and covering the area with a hydrophobic surface. Toxicity of an active phorbol ester is inversely proportional to the intrinsic reactivity of the ester linkage. Phorbol esters bearing free acid chains can directly activate PKC-δ but jatropha phorbol esters are restricted by their acid-moiety ring formations, suggesting similar mechanism of interaction with other phorbol-ester protein targets.

Key words: Phorbol ester, toxicity, protein kinase C, hydrophobic cover, intrinsic reactivity

1. Introduction

Toxicity of phorbol esters has received a significant amount of scientific attention, but the exact structure-activity relationships have been elusive. Although critical binding groups and certain structural requirements were determined, a satisfactory explanation of experimental observations remained in the knowledge gap.[1, 2]

Presented here are findings from computational analysis that reveal the binding mechanisms between phorbol esters and protein kinase C-delta (PKC-δ), leading to toxicity or lack of it.

1.1 Structure of Phorbol Esters

Naturally occurring phorbol esters have a similar alcohol moiety but different varieties for their carboxylic acid portions. However, there are two main versions of the alcohol groups, referred here as *12,13-* and *13,16- phorbols*. Most phorbol esters that exist in nature have the same stereochemistry for their alcohol portions, as shown in Fig. 1.[3] Note that the -OH group at C4 is in the beta (β) position (R-configuration).

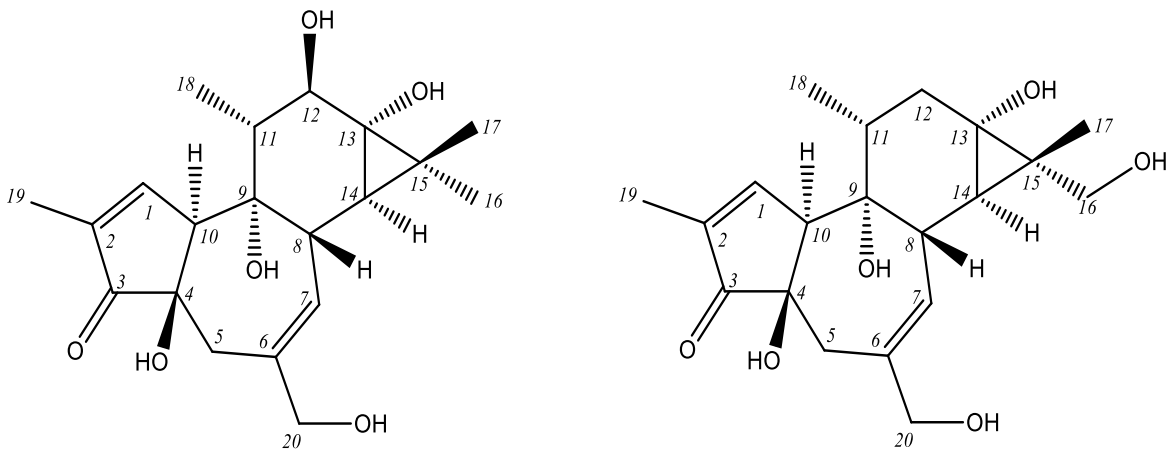
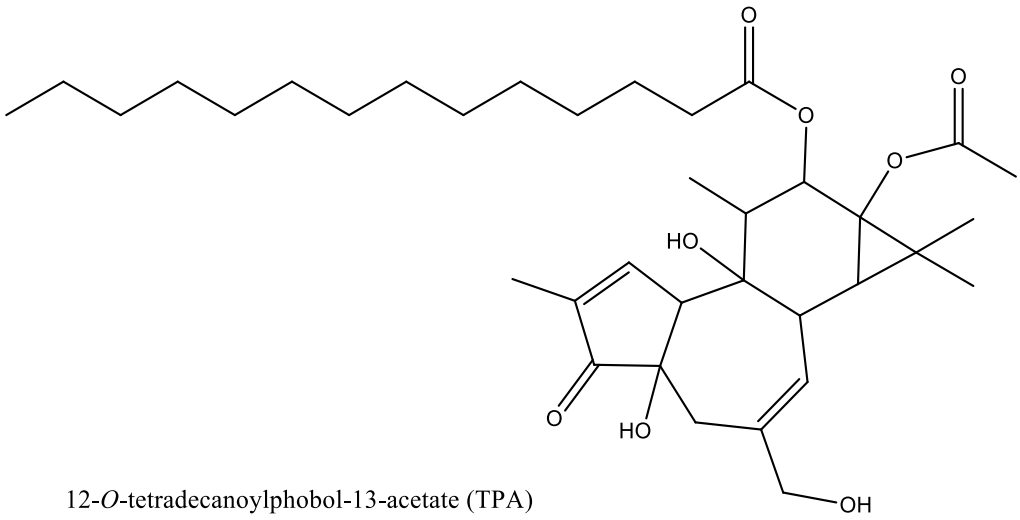
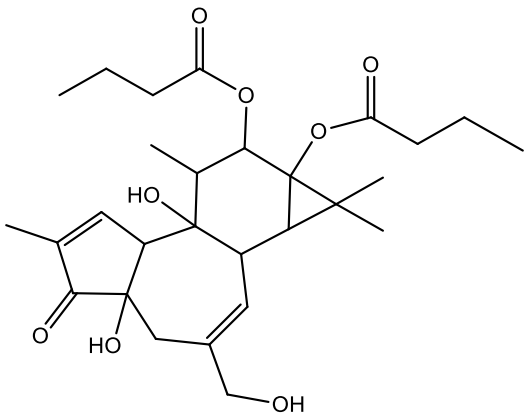


Fig. 1 Stereochemistry of alcohol moieties of naturally occurring phorbol esters

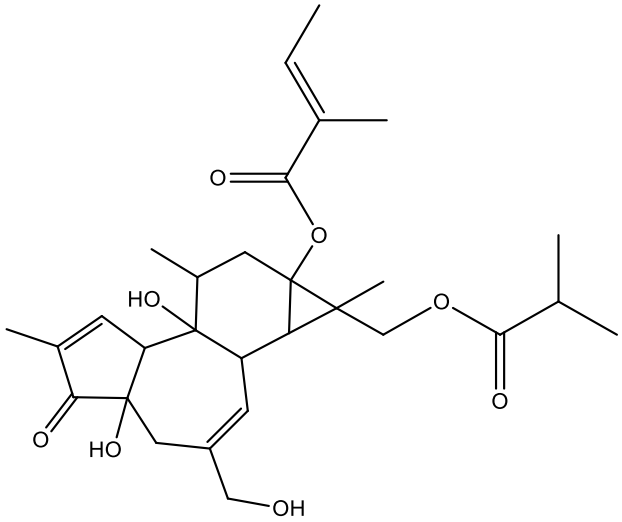
Examples of *12,13*- and *13,16*- phorbol esters are given in Fig. 2.[4–7] Jatropha factors 3, 4 and 5 are among the six phorbol esters, found in *Jatropha curcas*, all with their two acid tails subsequently joined into one.



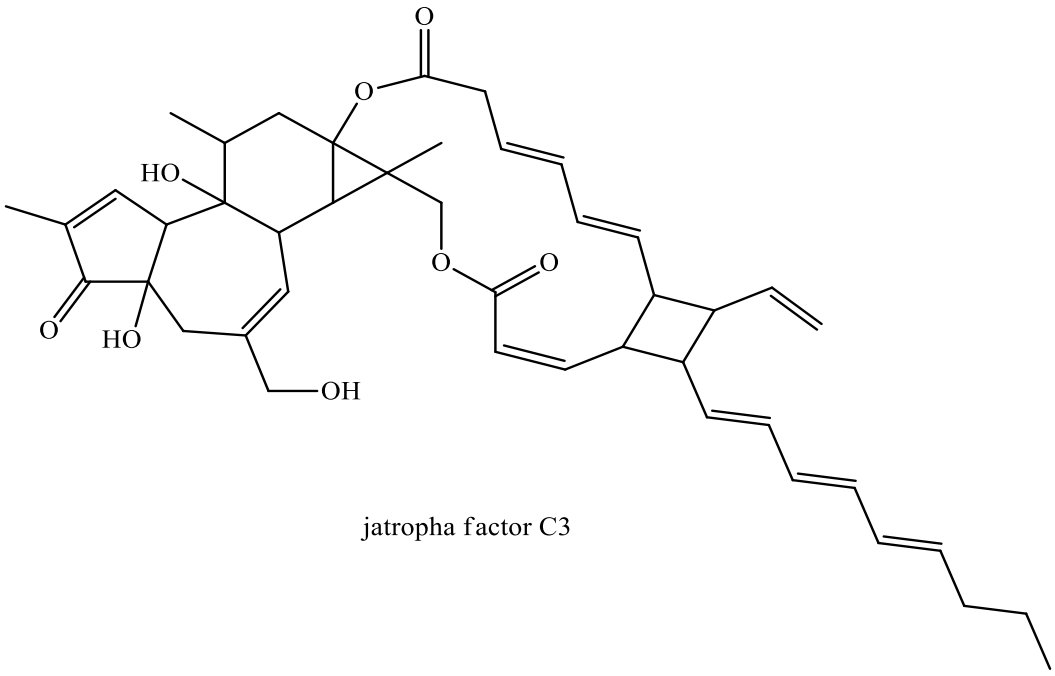
12-*O*-tetradecanoylphobol-13-acetate (TPA)



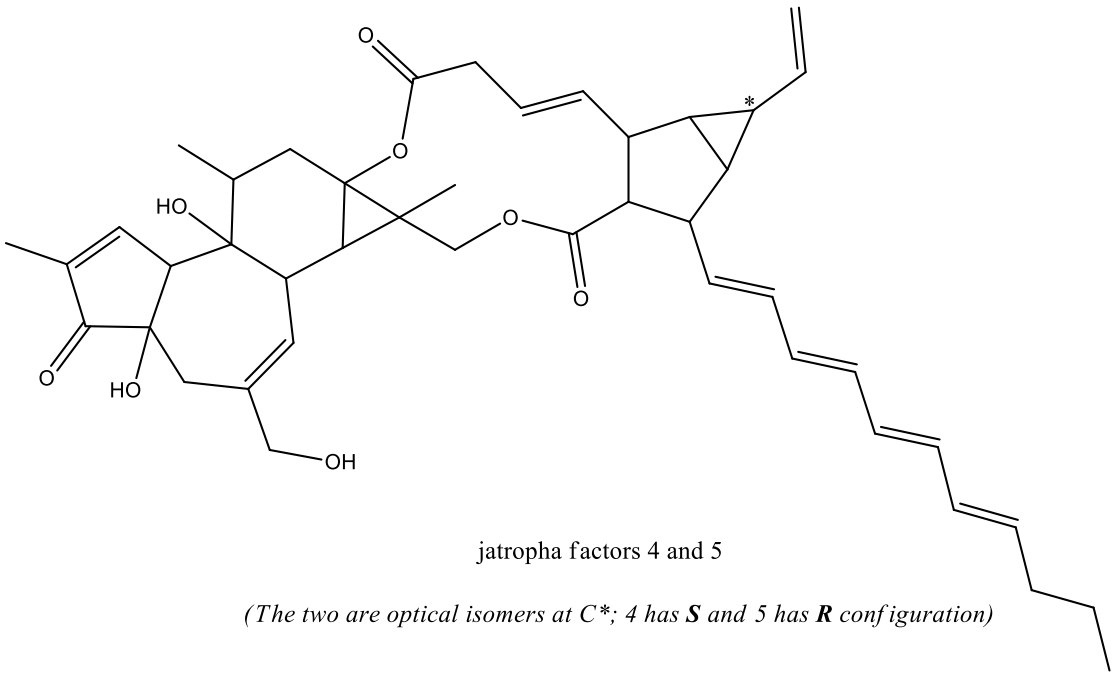
phorbol-12,13-dibutyrate (PDBu)



12-deoxyphorbol-13-tigliate-16-isobutyrate



jatropha factor C3



jatropha factors 4 and 5

(The two are optical isomers at C; 4 has **S** and 5 has **R** configuration)*

Fig. 2 Examples of two types of phorbol esters

1.2 Toxicity of Phorbol Esters

Although phorbols are non-toxic, their esters have exhibited high levels of harmfulness in animals. Oral dosages of phorbol esters have demonstrated toxicity to humans [8], cattle [9], goats [10], rabbits [11] and aquatic species [12]. Typically, LD_{50} of 27mg/kg body mass in mice was reported for *J. curcas* phorbol esters.[13]

The major biological target of phorbol esters is protein kinase C (PKC), a family of enzymes playing a pivotal part in signal transduction and regulation of cell differentiation.[14] Other phorbol-ester targets are chimaerins [15], Munc13 [16] and RasGRPs [17]; they have so far received limited attention. All these target proteins share a common feature, the C1 domain that carries the phorbol-ester binding site.[18] During normal signal transduction, PKC is activated by its interaction with diacylglycerol (DAG), and an acidic phospholipid, inside the cell; the complex then migrates and binds to specific membrane proteins. DAG is released soon after its hydrolysis.[19, 20] Experiments confirmed that only *S*-DAG has the ability to activate PKC; the other isomer, *R*-DAG is inactive.[21] There are three groups of PKC isoforms: lipid-sensitive, Ca^{2+}-dependent conventional or cPKC (comprising PKC-α, β_1, β_2 and γ), lipid-sensitive, Ca^{2+}-independent novel or nPKC (constituting PKC-δ, ε, η and θ) and atypical or aPKC (namely PKC-ξ and λ/ι) that neither bind DAG nor phorbol esters.[22, 23]

Phorbol esters activate PKC by binding irreversibly to the protein, thereby causing cell damage. The protein-phorbol ester complex is permanently inserted into the cell membrane because the phorbol ester does not metabolize easily.[24]

1.3 Structure-Activity Relationship

The stereochemistry of all the active phorbol esters, with C4-OH group in the β-position, show that the five membered ring is *trans*-linked to its adjacent seven membered ring, while the cyclohexane ring is *cis*-linked to the cyclopropane ring.[4]

Early studies on structural determinants of phorbol-ester activity suggested the oxygens at C3, C4, C9 and C20, as critical for binding to PKC.[25, 26] In 1995, Zhang *et al.* determined a crystal structure of a complex between phorbol-13-acetate and PKC-δ, showing hydrogen bonds involving C3, C4 and C20 oxygens of the phorbol ester.[27] Wang *et al.* later demonstrated that the C20 hydroxyl group contributed about 40% of the total hydrogen bonding energy.[5] The inactive α-C4-OH phorbol esters have a *cis*-conformation between the cyclopentane and cycloheptane rings, a set-up that shifts important binding groups from strategic positions.[28] Krauter *et al.* argued that a C13 acyl group played a significant role in the activity of phorbol esters.[29]

Other investigations had observed that hydrophobicity of the phorbol ester tail was an important factor for activation [30, 31], whose suggested mechanism involved closure of the hydrophilic PKC active site by the phorbol ester tail, then migration of the complex to the cell membrane.[27] That also explains why phorbols, with OH groups at positions C12, C13 or C16, are inactive.

Itai *et al.* performed molecular modelling on the active compounds, teleocidin and benzolactam, which are not phorbol esters, with PKC-δ and concluded that binding interactions do not necessarily involve the same protein groups, even if they bind competitively.[32] Endo *et al.* suggested that hydrophobic interactions had an influence in the binding of the ligand to PKC.[33] In their review of computer modelling methods, Mobley and Dill argued that a ligand may bind to a protein in multiple orientations and many other factors, among them ions, waters and solvation entropies could play significant roles.[34] Rahman and Das modelled structural determinants for interaction of DAG or phorbol esters to their target site and concluded that binding pocket volume and surface area do not have an effect on affinity.[35] Amino acid residues critical in the binding of phorbol esters to PKC-θ were identified by Czikora *et al.*[36] Molecular dynamics studies performed by Thangsunan *et al.*, on the binding interactions between PKC-δ and phorbol-13-acetate, indicated that the protein-ligand complex remained stable without significant structural changes for not less than 80 nanoseconds.[37]

However, some experimental observations bear testimony of the limitations of existing knowledge to determine structure-activity relationships in phorbol esters.

Hecker and Schmidt reported contrasting biological activities of two phorbol esters, whose long and short acid chains are on opposite sides. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) recorded beyond ten times tumor-promoting activity (82% against 8%) compared with 12-*O*-acetylphorbol-13-decanoate.[38] Another interesting set of results was published by Bertolini *et al*., who synthesized different phorbol esters with polar tail ends. Besides the general finding that polarity of short tails hindered activation of PKC, there were some long-tailed phorbol esters, whose activities showed no relationship with their structures. In Fig. 3, phorbol-ester structures and their K_D values are given. *(K_D is the phorbol-ester concentration required to move 50% of PKC to the lipid bilayer*) As a benchmark, K_D for TPA was 0.025μM.[20]

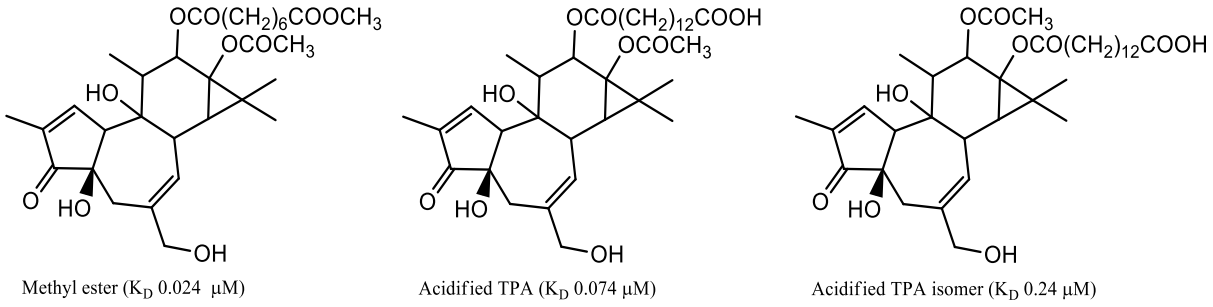


Fig. 3 Structures of Phorbol-Ester Derivatives and their Activities

Experiments by Kinghorn *et al*. on three phorbol esters showed effective mortality doses (ED_{50}) against *Artermia salina* of 3.8, 6.8 and 11.8 mg/l, to indicate the respective order of toxicity as TPA > phorbol-12,13-didecanoate > phorbol-12,13-dibenzoate.[39] Baird and Boutwell had reported the same order of tumor-promoting activity in mouse skin, but classified phorbol-12,13-diacetate as a very weak promoter (less than 1% that of TPA).[40, 41] However, this order was slightly different from *in vitro* activation of PKC found by

Castagna *et al*., which was TPA (100%) = phorbol-12,13-dibenzoate (100%) > phorbol-12,13-dibutyrate (88%) > phorbol-12,13-didecanoate (81%) > phorbol-12-tetradecanoate = phorbol-13-acetate = phorbol = 4α-phorbol-12,13-didecanoate = 0%.[42]

Bioassays of jatropha phorbol esters on snails (*Physa fontinalis*) and brine shrimp (*A. salina*) were studied by Roach *et al*. The order of potency in snails was jatropha factors C4 & C5 > jatropha factor C1 > jatropha factor C2 > jatropha factor C3 mixture, with EC_{50} values of 2.18, 4.12, 6.54 and 6.78 μg/l, respectively. A similar order was obtained against brine shrimp, with a switch in positions of the last two.[43]

Ellis *et al.* found out that some inactive phorbol esters could bind to PKC and inhibit the potent compounds.[44] Similar findings were reported for monoesters 12-deoxyphorbol-13-phenyl acetate (DPP) and 12-deoxyphorbol-13-acetate (prostratin).[45–47]

2. Computational Details

Computer modeling was performed using Maestro 10.2, a graphical user interface of the Schrödinger Suite's computational programs. The force field used in calculations was OPLS3.[48].

2.1 P450 Site of Metabolism

Individual phorbol esters were drawn and energy minimized. Prediction of sites of metabolism and intrinsic reactivity were done using Glide and Prime software that formed part of the Shrödinger Suite.[49] The 3A4 isoform of cytochrome P450 enzymes was chosen for the calculations, as it metabolizes over 50% of known xenobiotic compounds.[50] The P450 Site of Metabolism combines a linear free energy approach with induced-fit docking, to determine intrinsic reactivity of an atom and its accessibility to the reactive heme center of the P450 enzyme. Hammett and Taft scheme is used predict the intrinsic reactivity of an atom, as the sum of its baseline reactivity rate and connectivity factors. [51]

2.2 Molecular Docking

PKC-δ, co-crystalized with phorbol-13-acetate [27], was imported from the Protein Data Bank as 1PTR and was prepared using Protein Preparation Wizard of the Schrödinger Suite. Default settings plus filling in of missing side-chains and loops were employed, in the preprocessing stage. Using default settings for the rest of the protein preparation stages, dihedral angles were adjusted until the protein displayed the same geometry as the imported co-crystalized structure.[52]

Phorbol esters with experimentally determined activities, as well as DAG isomers, were drawn in Maestro and prepared by LigPrep from Schrödinger Suite – here computations were done with chiralities determined from 3D structures. All the other parameters were on default settings.[53]

The protein grid generation and the actual docking with the various types of ligands were performed using Glide software, also from Schrödinger Suite. For receptor grid generation, the size of the site was set at a maximum value of 25Å, but the other parameters remained on default settings. Precision settings for docking were on XP (extra precision) and the output included “per-residue interaction scores”.[49]

Ligand-protein interactions were analyzed by viewing the poses and determining hydrogen-bond strengths.

Docking of normal DAG molecules to PKC-δ was made difficult by the hydrophobic tails that stretched apart, during energy minimization of the molecule. This linear structure, without a polar head and non-polar tails, would not enter into the binding pocket. To circumvent this problem, we joined the two tails by introducing an artificial bond, about half-way along their lengths. Although the modification is fabricated, it gives a correct representation of DAG conformation inside the cell. After all, its chains can vary.[54]

2.3 Torsion Scan of C12 Ester Linkage

A rapid torsion scan was done for the C12 ester linkage of all 12,13-phorbol esters tested. This was carried out on Maestro, to determine energy barriers resulting from rotation of that specific bond.[48]

3. Results and Discussion

3.1 Determinants of Activation

Fig. 4 shows a computer model of PKC-δ binding site.

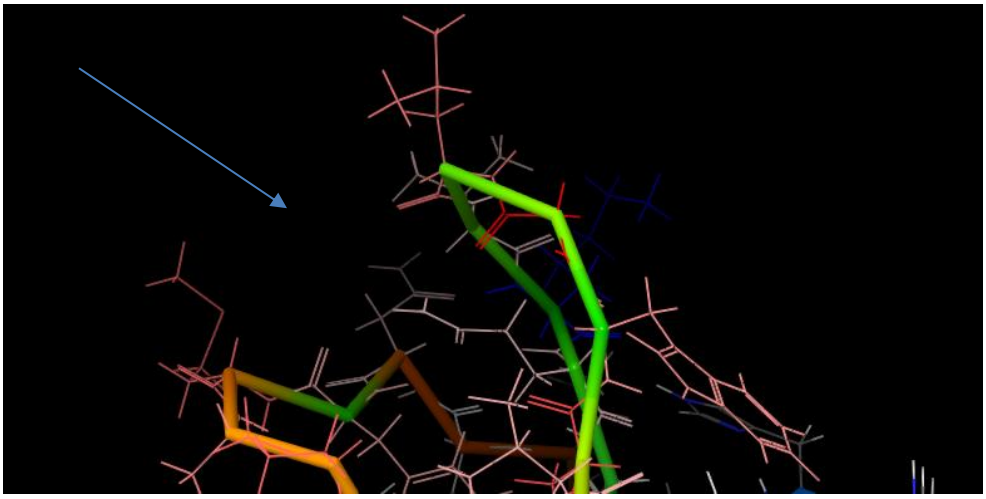


Fig. 4 Hydrophilic binding site of PKC-δ, as shown by the arrow

The amino acid residues mainly responsible for the hydrophilicity of this binding groove are glycine (*Gly-253*), leucine (*Leu-251*) and threonine (*Thr-242*), as demonstrated by Zhang *et al.* [27] and illustrated in Fig. 5. Other amino acids that contribute to the hydrophilic zone are also featuring in the same figure. The exterior surface of the binding site is covered by protruding hydrophobic groups.

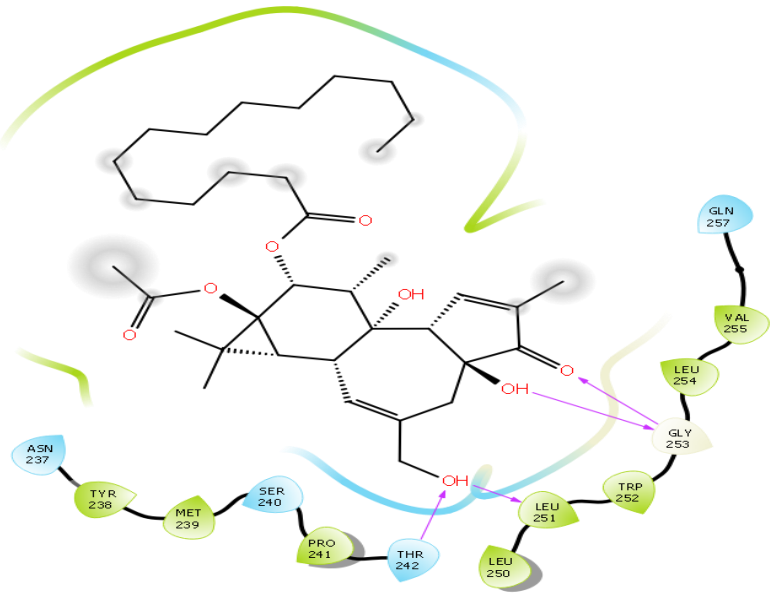


Fig. 5 Protein-Ligand Interaction Diagram between PKC-δ and TPA

Activation of PKC involves attachment of the ligand to the protein and then transfer of the complex to the cell membrane. All active and some inactive compounds bind to the same site and interact with the same amino-acid residues. Binding energies for all compounds that attach to the active site are similar too (see Table 1). The active S-DAG molecule and each of the following inactive compounds had total hydrogen-bond energy of -3 kcal/mol, when docked with PKC-δ: phorbol, phorbol-13-acetate and phorbol-12-tetradecanoate. It means that the pharmacophore for binding to the active site is the apt positioning of C3, C4 and C20 oxygen groups for phorbol esters, or equivalent for other compounds.

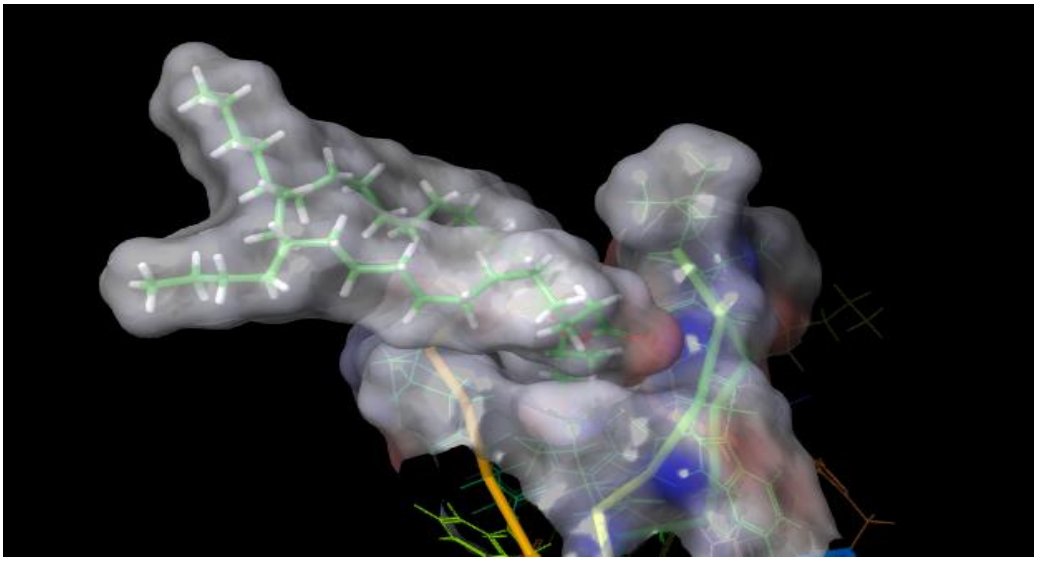
Table 1. Bond Energies, Torsion Energies & Activities for Different Phorbol Esters

Compound	***H-bond with PKC-δ (kcal/mol)***	***C-12 ester bond Torsion energy (MJ/mol @ -30°)***	***Experimental % PKC Activity **** [20, 38, 39, 41, 42]
TPA	-3.0	700	100

Methyl ester	-3.1	120	104
Phorbol-12, 13-dibutyrate	-3.2	80	88
Phorbol-12, 13-didecanoate	-3.3	5	69
Phorbol-12, 13-dibenzoate	-3.0	15	66
Acidified TPA	-3.4	30	34
Acidified TPA isomer	-3.1	240	10
12-*O*-Acetylphorbol-13-decanoate	-3.0	370	10
Phorbol-12,13-diacetate	-3.0	370	< 1

**Values stated with reference to TPA, whose activity was pegged at 100%*

Binding to the active site is not a sufficient condition for activation of PKC; an active compound should cover the area with a hydrophobic surface. Fig. 6 exhibits a distinction between an activator, S-DAG, and non-activating phorbol-13-acetate, using electrostatic potentials of their binding modes. Phorbol-13-acetate does not activate PKC-δ because its C12-OH group is exposed to the exterior surface of the protein-ligand complex.



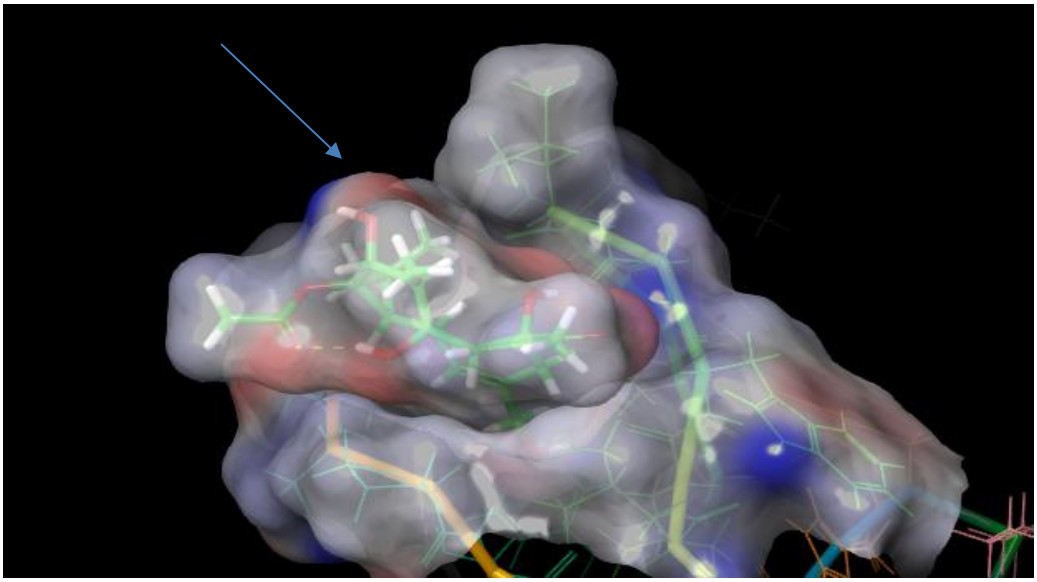


Fig. 6 Binding poses for S-DAG (top) and phorbol-13-acetate (bottom)

Phorbol is not active because the two -OH groups at C12 and C13 positions protrude outwards of the complex, while phorbol-12-tetradecanoate is betrayed by the C13 hydroxyl group. Since such compounds bind to the protein with equal strength as activators, it explains why they were experimentally found to compete with the potent substances for the same site.[44] Likewise, phorbol-12,13-diacetate, prostratin and DPP bind well to PKC-δ but expose their hydrophilic ester linkages, hence, they fail to activate the protein. The reason for the negligible activity of these compounds has been revealed by computer modelling.

The presence of a hydrophilic moiety on the tail does not necessarily mean loss of activity for the phorbol ester. Some of the structures synthesized by Bertolini *et al.*[20] were docked with PKC-δ and the polar tip would attach at a point away from the binding site, as the tail flipped to the side. It means that closure of the PKC binding site with a hydrophobic cover is a sufficient criterion for activation, which does not necessarily involve insertion of phorbol ester tail into the cell membrane.

3.2 Features Causing Binding Failure

R-DAG and αTPA did not dock into the binding groove of PKC-δ, in agreement with what has been reported in literature. Their oxygen centers, responsible for attaching to the critical amino acid residues, are pointing in wrong directions because of stereochemical differences.[21, 28]

All the six jatropha phorbol esters failed to lock into the PKC-δ binding site, with or without induced-fit docking. Even the reduction of molecular weight, to a similar magnitude as TPA, by cutting off part of the tail, did not improve the interaction. The ring structure that connects the two acid chains makes jatropha phorbol esters too bulky to fit into the binding site; see Fig. 7.

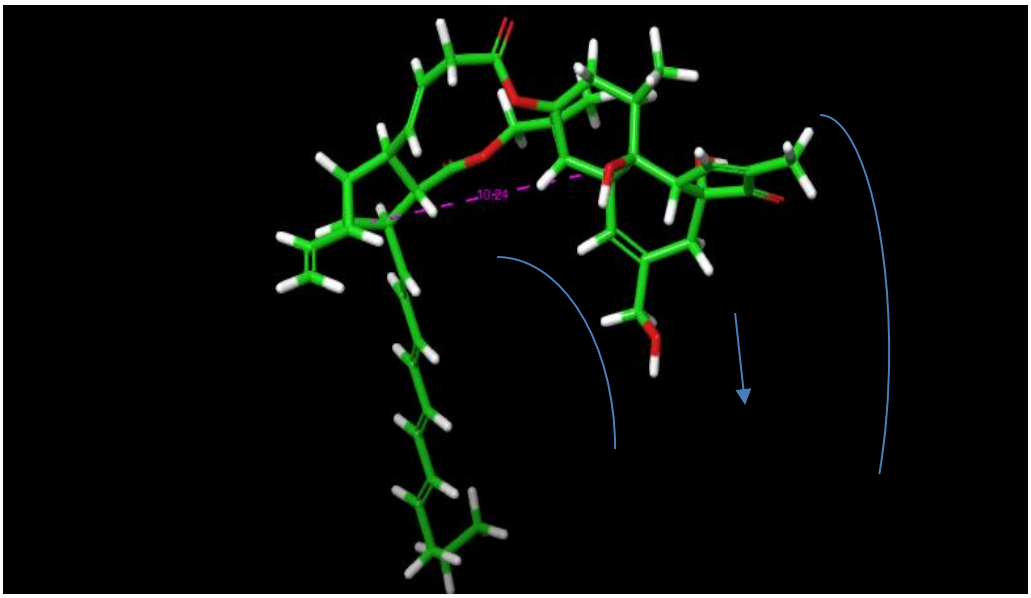


Fig. 7 Jatropha factor C5 unable to descent fully into the binding pocket

According to computational measurements, these ring structures, for all the jatropha phorbol esters, had diameters ranging from 9.9 to 10.9Å, against a binding site opening of 8.6Å. Having said that, there is experimental evidence for toxicity of jatropha phorbol esters.[43] Hence, activities of those molecules are most probably linked to phorbol-ester targets, other than PKC-δ. Stahelin *et al*. demonstrated that isoforms of PKC have different

binding affinities for DAG and phorbol esters, the reasons for which are still uncertain.[55] Our findings from computer modelling showed that jatropha phorbol esters are too big to fit into the PKC-δ binding site, hence they probably interact with other targets by the same mechanism. Attempts to model other phorbol-ester targets with jatropha phorbol esters were limited by the availability of in-silico, protein-ligand, co-crystallized structures. In fact, only one ligand-bound C1 domain has been reported so far.[2]

3.3 Determinants of Toxicity

The toxicity of an active compound lies in its resistance to metabolize. S-DAG is non-toxic because it is hydrolyzed, soon after translocating PKC to the cell membrane.[20] In that regard, the toxicity of a phorbol ester is expected to be inversely proportional to the intrinsic reactivity of an ester linkage. This intrinsic reactivity of an individual atom, combined with iron-accessibility at the heme of P450 enzyme, is indicated by a green circle, whose radius increases with the score (see Fig. 8). The scores are also given as values; the more positive the figure, the more reactive the atom.[51] In Fig. 8, a 12,13-phorbol ester (phorbol-12,13-dibenzoate) is compared with a 13,16-phorbol ester (12-deoxyphorbol-13-tigliate-16-isobutyrate), with respect to the intrinsic reactivities of their ester linkages.

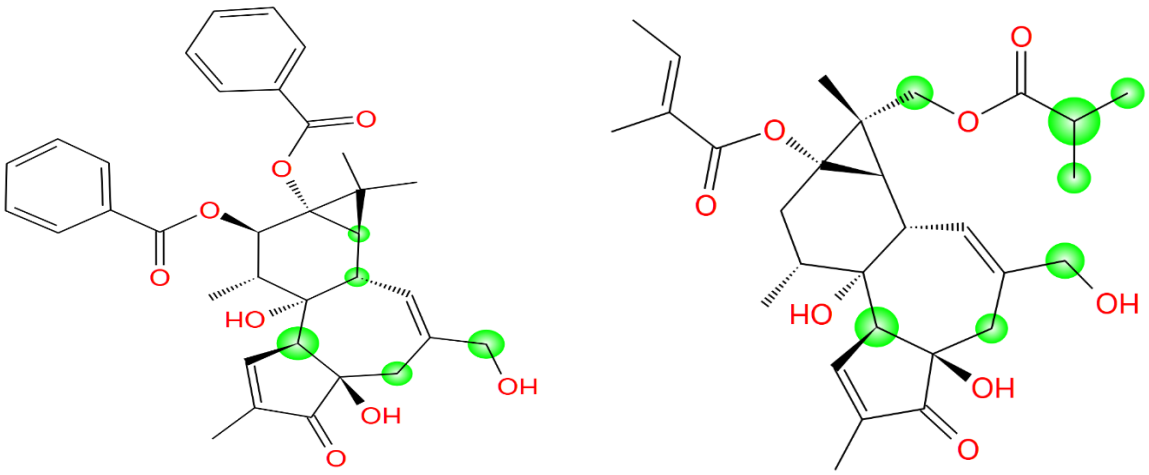


Fig. 8 Intrinsic Reactivities of a 12,13-Phorbol Ester against a 13,16-Phorbol Ester

Attention is given to the hydrolysis of acid tails as they are the major determinants of phorbol-ester toxicity. Like all the other 12,13-diesters, phorbol-12,13-dibenzoate has unreactive ester linkages, making the molecule difficult to hydrolyze. On the other hand, 13,16-diesters have a reactive C16 ester linkage, towards cytochrome P450 enzymes.

Table 2 lists intrinsic reactivities of the C16 ester linkage, for 13,16-phorbol esters, against their experimentally determined toxicities.

Table 2. Intrinsic Reactivity versus Toxicity of 13,16-Phorbol Esters

Phorbol Ester	***C13-linkage Intrinsic Reactivity***	***C16-linkage Intrinsic Reactivity***	***Snail bioassay, P. fontinalis, EC_{50} (μg/l)*** [43]
Jatropha factors C4/C5	-0.7	0.9	2.18
Jatropha factor C1	-0.6	0.9	4.12
Jatropha factor C2	-0.1	0.9	6.54
Jatropha factor C3	-0.0	1.9	6.78
Jatropha factor C6	0.2	1.9	not measured
12-Deoxyphorbol-13-tigliate-16-isobutyrate	-1.2	3.6	not measured

All 12,13-phorbol esters, the inactive included, recorded very low intrinsic reactivity values of between -2.8 and -3.9 on the C12 ester linkage and -2.3 to -4.1 on the C13 connection. What it means is that αTPA could exhibit the same toxicity as TPA, only if it managed to bind to the active site and expose a hydrophobic surface. Conversely, all 13,16-phorbol esters must be less toxic than their 12,13- counterparts, as is displayed in Table 2 that their calculated intrinsic reactivities are much higher. Although direct experimental evidence is not available, Roach *et al*., in their comparisons for platelet aggregation by phorbol esters, observed the following ED_{50} (μM) values: 0.5 (TPA), 1.8 (C4/C5), 4.4 (C1), 6.1 (C3) and 7.9 (C2). The order for jatropha esters agrees with that of their toxicity against

A. salina.[43] Hence, it could be extrapolated that TPA and other 12,13-phorbol esters are generally more toxic than 13,16-diesters.

Computational values of intrinsic reactivity in Table 2 provide evidence of a relationship with experimentally measured toxicities. A mixture of the epimers, jatropha factors C4 and C5 (Fig. 2), known to be more toxic than any other jatropha phorbol ester, had the least reactivity towards hydrolysis. Jatropha factor C6, though not measured can be predicted to be least toxic among the jatropha esters. Since 12-deoxyphorbol-13-tigliate-16-isobutyrate has several points prone to attack, around the C16 ester linkage, the figure with the highest magnitude was chosen; the reason is that hydrolysis around that region will detoxify the molecule. This compound is expected to be less toxic than jatropha phorbol esters.

What causes differences in toxicity among the 12,13-phorbol esters, if their intrinsic reactivities are the same? We observed that the answer lies in the rotation of the C-O bond, at the C12 position. As the 12,13-phorbol esters approach the PKC-δ binding site, they do so with the C20 -OH group in front and the tails behind. The C13 ester linkage attaches to the lower lip of the binding entrance, while the C12 tail lies along the upper edge. Fig. 9 illustrates a PKC-TPA complex, in which the C12 ester bond is restricted from twisting to the right (positive direction) by the protein surface. Freedom of rotation is in the negative direction, when the C12 tail can be moved to the left.

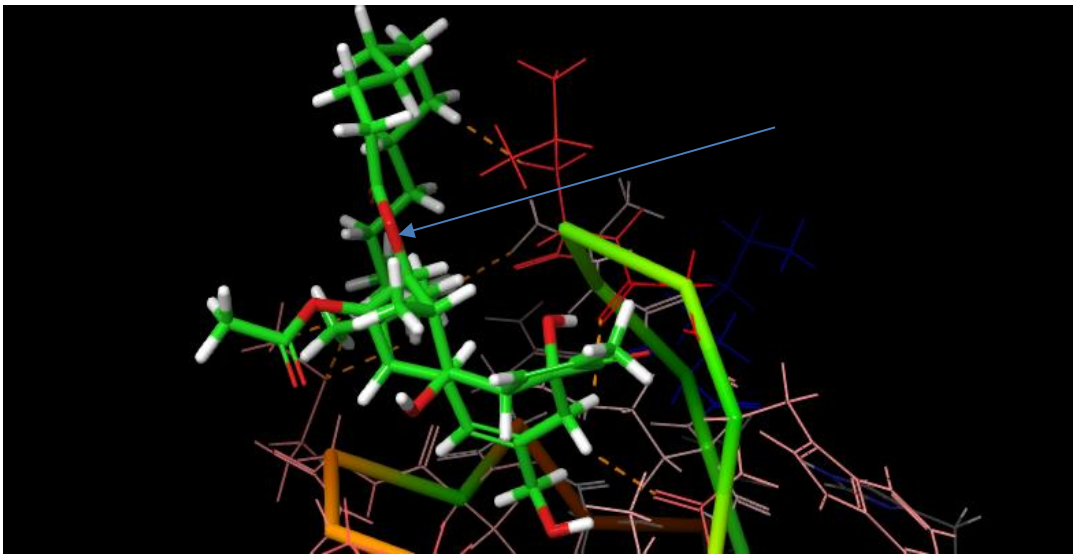


Fig. 9 Toxic TPA complexed with PKC-δ (C12 ester bond shown by arrow)

Results of rapid torsion scan of the C_{12}-O bond are presented in Table 1. Before the scan was done, the C_{12}-O and C_{13}-O bonds were rotated accordingly to achieve a zero-point phorbol-ester conformation exhibited in Fig. 9. Torsion energies given in Table 1 refer to the amount of resistance the C12 tail would face when rotated from zero point by -30° (to the left). TPA recorded an energy of 700 MJ/mol, to declare that its conformation is stable. Once the molecule is bound to the PKC, it almost always locks into a position, which in this case ensures hydrophobicity and toxicity. In contrast, 12-*O*-acetylphorbol-13-decanoate, with an energy of 370 MJ/mol also maintains a fixed orientation, but exposing the C12 oxygen group, to exhibit inactivity. Compounds with low energy barriers, such as phorbol-12,13-dibenzoate and phorbol-12,13-didecanoate can exhibit varying levels of toxicities, depending on whether or not they expose a hydrophilic oxygen group. In fact, their toxicity values noted in Table 1 are averages of 100% [42] and 32% [39] for the dibenzoate and 81% [42] and 56% [39] for the didecanoate. The meaning of this is that toxicity of a phorbol ester becomes probabilistic when the C12 rotational energy barrier is reduced.

4. Conclusion

The determination of mechanisms of phorbol-ester toxicity is a complex process, involving unrelated aspects, at times. In some cases, it is easy to tell the features that give rise to toxicity, but in other instances the signs are not obvious. This task has been accomplished by computer modelling. Two types of phorbol esters have been discussed: 12,13- and 13,16-diesters. Their toxicity arises from their binding to a phorbol-ester target protein, such as PKC.

Toxic phorbol esters have a specific arrangement of oxygen groups that they use to bind to the active site of the target protein. To activate the protein, the phorbol ester should close

the binding pocket with a hydrophobic surface. Once the protein is activated, the phorbol ester should resist metabolism for it to exhibit toxicity. The 12,13-phorbol esters are more difficult to hydrolyze, hence more toxic than 13,16-diesters. Since 12,13-phorbol esters are equally unreactive, their distinctions in toxicity are caused by different conformational stabilities. A compound that resists rotation of the C12 acid tail is conformationally stable and maintains constant level of toxicity, while the one allowing bond rotation exhibits varying toxicities.

Intrinsic reactivity of jatropha phorbol esters is not the same and their ease of hydrolysis is inversely proportional to their toxicities. They were, nevertheless found to be too big to bind to PKC-δ. Their toxicity is expected to arise from their interactions with other target proteins, by the same mechanism as 12,13-diesters.

5. References

1. Wakandigara, A., Nhamo, L. R. M., & Kugara, J. (2013). Chemistry of Phorbol Ester Toxicity in Jatropha curcas Seed – a Review. *International Journal of Biochemistry Research & Review, 3*(3), 146–161. https://doi.org/10.9734/IJBCRR/2013/2956
2. Das, J., & Rahman, G. M. (2014). C1 Domains: Structure and Ligand-Binding Properties. *Chemical Reviews, 114*(24), 12108–12131. https://doi.org/10.1021/cr300481j

3. El-sherei, M. M., Islam, W. T., El-Dine, R. S., El-Toumy, S. A., & Ahmed, S. R. (2015). Phytochemical investigation of the cytotoxic latex of Euphorbia cooperi N.E.Br. *Australian Journal of Basic and Applied Sciences*, *9*(11), 488–493.
4. Goel, G., Makkar, H. P. S., Francis, G., & Becker, K. (2007). Phorbol Esters: Structure, Biological Activity, and Toxicity in Animals. *International Journal of Toxicology*, *26*(4), 279–288. https://doi.org/10.1080/10915810701464641
5. Wang, S., Kazanietz, M. G., Blumberg, P. M., Marquez, V. E., & Milne, G. W. A. (1996). Molecular Modeling and Site-Directed Mutagenesis Studies of a Phorbol Ester-Binding Site in Protein Kinase C. *Journal of Medicinal Chemistry*, *39*(13), 2541–2553. https://doi.org/10.1021/jm950403n
6. Gundidza, M., Sorg, B., & Hecker, E. (1992). A skin irritant phorbol ester from Euphorbia cooperi N E Br. *Central African Journal of Medicine*, *38*(12), 444–447.
7. Haas, W., Sterk, H., & Mittelbach, M. (2002). Novel 12-Deoxy-16-hydroxyphorbol Diesters Isolated from the Seed Oil of Jatropha curcas. *Journal of Natural Products*, *65*(10), 1434–1440. https://doi.org/10.1021/np020060d
8. Becker, K., & Makkar, H. P. S. (1998). Effects of Phorbol Esters in Carp (Cyprinus carpio L). *Veterinary and Human Toxicology*, *40*(2), 82–86.
9. Ahmed, O. M. M., & Adam, S. E. I. (1979). Effects of Jatropha curcas on Calves. *Veterinary Pathology*, *16*(4), 476–482. https://doi.org/10.1177/030098587901600411
10. Adam, S. E. I., & Magzoub, M. (1975). Toxicity of Jatropha curcas for goats. *Toxicology*, *4*, 347–354.

11. Gandhi, V. M., Cherian, K. M., & Mulky, M. J. (1995). Toxicological studies on ratanjyot oil. *Food and Chemical Toxicology*, *33*(1), 39–42. https://doi.org/10.1016/0278-6915(95)80246-0

12. Devappa, R. K., Rajesh, S. K., Kumar, V., Makkar, H. P. S., & Becker, K. (2012). Activities of Jatropha curcas phorbol esters in various bioassays. *Ecotoxicology and Environmental Safety*, *78*, 57–62. https://doi.org/10.1016/j.ecoenv.2011.11.002

13. Li, C.-Y., Devappa, R. K., Liu, J.-X., Lv, J.-M., Makkar, H. P. S., & Becker, K. (2010). Toxicity of Jatropha curcas phorbol esters in mice. *Food and Chemical Toxicology*, *48*(2), 620–625. https://doi.org/10.1016/j.fct.2009.11.042

14. Newton, A. C. (2018). Protein kinase C: perfectly balanced. *Critical Reviews in Biochemistry and Molecular Biology*, *53*(2), 208–230. https://doi.org/10.1080/10409238.2018.1442408

15. Wang, H., & Kazanietz, M. G. (2002). Chimaerins, Novel Non-protein Kinase C Phorbol Ester Receptors, Associate with Tmp21-I (p23): Evidence for a Novel Anchoring Mechanism Involving the Chimaerin C1 Domain. *Journal of Biological Chemistry*, *277*(6), 4541–4550. https://doi.org/10.1074/jbc.M107150200

16. Kang, L., He, Z., Xu, P., Fan, J., Betz, A., Brose, N., & Xu, T. (2006). Munc13-1 is required for the sustained release of insulin from pancreatic β cells. *Cell Metabolism*, *3*(6), 463–468. https://doi.org/10.1016/j.cmet.2006.04.012

17. Lorenzo, P. S., Kung, J. W., Bottorff, D. A., Garfield, S. H., Stone, J. C., & Blumberg, P. M. (2001). Phorbol Esters Modulate the Ras Exchange Factor RasGRP3. *Cancer Research*, *61*, 943–949.

18. Kazanietz, M. G. (2002). Novel "Nonkinase" Phorbol Ester Receptors: The C1 Domain Connection. *Molecular Pharmacology*, *61*(4), 759–767. https://doi.org/10.1124/mol.61.4.759
19. Webb, B. L. J., Hirst, S. J., & Giembycz, M. A. (2000). Protein kinase C isoenzymes: a review of their structure, regulation and role in regulating airways smooth muscle tone and mitogenesis. *British Journal of Pharmacology*, *130*(7), 1433–1452. https://doi.org/10.1038/sj.bjp.0703452
20. Bertolini, T. M., Giorgione, J., Harvey, D. F., & Newton, A. C. (2003). Protein Kinase C Translocation by Modified Phorbol Esters with Functionalized Lipophilic Regions. *Journal of Organic Chemistry*, *68*(13), 5028–5036. https://doi.org/10.1021/jo030029w
21. Boni, L. T., & Rando, R. R. (1985). The Nature of Protein Kinase C Activation by Physically Defined Phospholipid Vesicles and Diacylglycerols*. *Journal of Biological Chemistry*, *260*(19), 10819–10825.
22. Steinberg, S. F. (2008). Structural Basis of Protein Kinase C Isoform Function. *Physiological Reviews*, *88*(4), 1341–1378. https://doi.org/10.1152/physrev.00034.2007
23. Eichmann, T. O., & Lass, A. (2015). DAG tales: the multiple faces of diacylglycerol—stereochemistry, metabolism, and signaling. *Cellular and Molecular Life Sciences*, *72*(20), 3931–3952. https://doi.org/10.1007/s00018-015-1982-3
24. Rando, R. R. (1988). Regulation of protein kinase C activity by lipids. *The FASEB Journal*, *2*(8), 2348–2355. https://doi.org/10.1096/fasebj.2.8.3282960
25. Jeffrey, A. M., & Liskamp, R. M. (1986). Computer-assisted molecular modeling of tumor promoters: rationale for the activity of phorbol esters, teleocidin B, and aplysiatoxin. *Proceedings of the National Academy of Sciences*, *83*(2), 241–245. https://doi.org/10.1073/pnas.83.2.241

26. Wender, P. A., Cribbs, C. M., Koehler, K. F., Sharkey, N. A., Herald, C. L., Kamano, Y., … Blumberg, P. M. (1988). Modeling of the bryostatins to the phorbol ester pharmacophore on protein kinase C. *Proceedings of the National Academy of Sciences*, *85*(19), 7197–7201. https://doi.org/10.1073/pnas.85.19.7197

27. Zhang, G., Kazanietz, M. G., & Blumberg, P. M. (1995). Crystal Structure of the Cys2 Activator-Binding Domain of Protein Kinase C8 in Complex with Phorbol Ester. *Cell*, *81*, 917–924.

28. Silinsky, E. M., & Searl, T. J. (2003). Phorbol esters and neurotransmitter release: more than just protein kinase C? *British Journal of Pharmacology*, *138*(7), 1191–1201. https://doi.org/10.1038/sj.bjp.0705213

29. Krauter, G., Lieth, C.-W., Schmidt, R., & Hecker, E. (1996). Structure/Activity Relationships of Polyfunctional Diterpenes of the Tigliane Type. A Pharmacophore Model for Protein-Kinase-C Activators Based on Structure/Activity Studies and Molecular Modeling of the Tumor Promoters 12-O-Tetradecanoylphorbol 13-Acetate and 3-O-Tetradecanoylingenol. *European Journal of Biochemistry*, *242*(2), 417–427. https://doi.org/10.1111/j.1432-1033.1996.0417r.x

30. Hecker, E. (1968). Cocarcinogenic Principles from the Seed Oil of Croton tiglium and from Other Euphorbiaceae. *Cancer Research*, *28*, 2338–2349.

31. Sharkey, N. A., & Blumberg, P. M. (1985). Highly Lipophilic Phorbol Esters as Inhibitors of Specific [3 H]Phorbol 12,13-Dibutyrate Binding. *Cancer Research*, *45*, 19–24.

32. Itai, A., Matsuo, A., Mizutani, M. Y., Tomioka, N., Shitaka, M. T., Endo, Y., & Shudo, K. (1997). Advanced Computational Docking of Two Teleocidin Congeners to Cys2 Domain of Protein Kinase Cd. *Chemical and Pharmaceutical Bulletin*, *45*(3), 573–575.

33. Endo, Y., Takehana, S., Ohno, M., Driedger, P. E., Stabel, S., Mizutani, M. Y., … Shudo, K. (1998). Clarification of the Binding Mode of Teleocidin and Benzolactams to the Cys2 Domain of Protein Kinase Cδ by Synthesis of Hydrophobically Modified, Teleocidin-Mimicking Benzolactams and Computational Docking Simulation. *Journal of Medicinal Chemistry*, *41*(9), 1476–1496. https://doi.org/10.1021/jm970704s

34. Mobley, D. L., & Dill, K. A. (2009). Binding of Small-Molecule Ligands to Proteins: "What You See" Is Not Always "What You Get." *Structure*, *17*(4), 489–498. https://doi.org/10.1016/j.str.2009.02.010

35. Rahman, G. M., & Das, J. (2015). Modeling studies on the structural determinants for the DAG/phorbol ester binding to C1 domain. *Journal of Biomolecular Structure and Dynamics*, *33*(1), 219–232. https://doi.org/10.1080/07391102.2014.895679

36. Czikora, A., Pany, S., You, Y., Saini, A. S., Lewin, N. E., Mitchell, G. A., … Das, J. (2018). Structural determinants of phorbol ester binding activity of the C1a and C1b domains of protein kinase C theta. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1860*(5), 1046–1056. https://doi.org/10.1016/j.bbamem.2018.01.007

37. Thangsunan, P., Tateing, S., Hannongbua, S., & Suree, N. (2016). Structural insights into the interactions of phorbol ester and bryostatin complexed with protein kinase C: a comparative molecular dynamics simulation study. *Journal of Biomolecular Structure and Dynamics*, *34*(7), 1561–1575. https://doi.org/10.1080/07391102.2015.1084479

38. Hecker, E., & Schmidt, R. (1974). Phorbolesters — the Irritants and Cocarcinogens of Croton Tiglium L. *Progress in the Chemistry of Organic Natural Products*, *31*, 377–467. https://doi.org/10.1007/978-3-7091-7094-6_7

39. Kinghorn, D. A., Harjes, K. K., & Doorenbos, N. J. (1977). Screening Procedure for Phorbol Esters Using Brine Shrimp (Artemia salina) Larvae. *Journal of Pharmaceutical Sciences*, *66*(9), 1362–1363. https://doi.org/10.1002/jps.2600660949

40. Baird, W. M., & Boutwell, R. K. (1971). Tumor-promoting Activity of Phorbol and Four Diesters of Phorbol in Mouse Skin. *Cancer Research*, *31*, 1074–1079.

41. Brynes, P. J., Schmidt, R., & Hecker, E. (1980). Plasminogen activator induction and platelet aggregation by phorbol and some of its derivatives: Correlation with skin irritancy and tumor-promoting activity. *Journal of Cancer Research and Clinical Oncology*, *97*(3), 257–266. https://doi.org/10.1007/BF00405777

42. Castagna, M., Kaibuchi, K., Kikkawa, U., & Nishizuka, Y. (1982). Direct Activation of Calcium-activated, Phospholipid-dependent Protein Kinase by Tumor-promoting Phorbol Esters. *Journal of Biological Chemistry*, *257*(13), 7847–7851.

43. Roach, J. S., Devappa, R. K., Makkar, H. P. S., & Becker, K. (2012). Isolation, stability and bioactivity of Jatropha curcas phorbol esters. *Fitoterapia*, *83*(3), 586–592. https://doi.org/10.1016/j.fitote.2012.01.001

44. Ellis, C. A., Brooks, S. F., Brooks, G., Tudor Evans, A., Morrice, N., Evans, Fred. J., & Aitken, A. (1987). The effects of phorbol esters with different biological activities on protein kinase C. *Phytotherapy Research*, *1*(4), 187–190. https://doi.org/10.1002/ptr.2650010412

45. Kazanietz, M. G., Krausz, K. W., & Blumberg, P. M. (1992). Differential Irreversible Insertion of Protein Kinase C into Phospholipid Vesicles by Phorbol Esters and Related Activators*. *Journal of Biological Chemistry*, *267*(29), 20878–20886.

46. Szallasi, I., Krsmanovic, L., & Blumberg, P. M. (1993). Nonpromoting 12-Deoxyphorbol 13-Esters Inhibit Phorbol 12-Myristate 13-Acetate Induced 1\imor Promotion in CD-I Mouse Skin. *Cancer Research*, *53*, 2507–2512.
47. Bögi, K., Lorenzo, P. S., Szallasi, Z., Acs, P., Wagner, G. S., & Blumberg, P. M. (1998). Differential Selectivity of Ligands for the Cla and Clb Phorbol Ester Binding Domains of Protein Kinase Cd: Possible Correlation with Tumor- promoting Activity. *Cancer Research*, *58*, 1423–1428.
48. Schrodinger, LLC. (2015). *Maestro* (Version 10.2.). New York, NY: Schrodinger, LLC.
49. Schrodinger, LLC. (2015). *Glide* (6.7.). New York, NY: Schrodinger, LLC.
50. Rowland, P., Blaney, F. E., Smyth, M. G., Jones, J. J., Leydon, V. R., Oxbrow, A. K., … Bridges, A. M. (2006). Crystal Structure of Human Cytochrome P450 2D6. *Journal of Biological Chemistry*, *281*(11), 7614–7622. https://doi.org/10.1074/jbc.M511232200
51. Schrodinger, LLC. (2012). *P450 Site of Metabolism* (2nd ed.). New York, NY: Schrodinger, LLC.
52. Schrodinger, LLC. (2012). *Protein Preparation Wizard*. New York, NY: Schrodinger, LLC.
53. Schrodinger, LLC. (2009). *LigPrep* (Version 2.3.). New York, NY: Schrodinger, LLC.
54. Wakelam, M. J. O. (1998). Diacylglycerol – when is it an intracellular messenger? *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1436*(1–2), 117–126. https://doi.org/10.1016/S0005-2760(98)00123-4
55. Stahelin, R. V., Kong, K.-F., Raha, S., Tian, W., Melowic, H. R., Ward, K. E., … Cho, W. (2012). Protein Kinase Cθ C2 Domain Is a Phosphotyrosine Binding Module That Plays a Key Role in Its Activation. *Journal of Biological Chemistry*, *287*(36), 30518–30528. https://doi.org/10.1074/jbc.M112.391557