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Chemical modifications of resveratrol for improved protein kinase C alpha activity

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ABSTRACT

Resveratrol (**1**) is a naturally occurring phytoalexin that affects a variety of human disease models, including cardio- and neuroprotection, immune regulation, and cancer chemoprevention. One of the possible mechanisms by which resveratrol affects these disease states is by affecting the cellular signaling network involving protein kinase C alpha (PKCα). PKCα is a member of the family of serine/threonine kinases, whose activity is inhibited by resveratrol. To study the structure–activity relationship, several monoalkoxy, dialkoxy and hydroxy analogs of resveratrol have been synthesized, tested for their cytotoxic effects on HEK293 cells, measured their effects on the membrane translocation properties of PKCα in the presence and absence of the PKC activator TPA, and studied their binding with the activator binding domain of PKCα. The analogs showed less cytotoxic effects on HEK293 cells and caused higher membrane translocation (activation) than that of resveratrol. Among all the analogs, **3**, **16** and **25** showed significantly higher activation than resveratrol. Resveratrol analogs, however, inhibited phorbol ester-induced membrane translocation, and the inhibition was less than that of resveratrol. Binding studies using steady state fluorescence spectroscopy indicated that resveratrol and the analogs bind to the second cysteine-rich domain of PKCα. The molecular docking studies indicated that resveratrol and the analogs interact with the protein by forming hydrogen bonds through its hydroxyl groups. These results signify that molecules developed on a resveratrol scaffold can attenuate PKCα activity and this strategy can be used to regulate various disease states involving PKCα.

1. Introduction

(*E*)-Resveratrol or (*E*)-1-(3,5-dihydroxyphenyl)-2-(4-hydroxyphenyl) ethene (**1**, Scheme 1), is a naturally occurring phytoalexin found in grapes, red wine, peanuts, olive oil, cranberries, and other food.[1,2] Numerous studies highlighted the effects of resveratrol in a variety of human disease models, including cardio- and neuroprotection, immune regulation, and cancer chemoprevention.[3–8] Some of the recent studies evaluated promising biological properties,[9,10] including antioxidant activity,[11] antiestrogenic activity[12] inhibition of cyclooxygenase,[13] inhibition of platelet aggregation.[14] Resveratrol showed chemopreventive activities against human degenerative diseases such as atherosclerosis[15] and cancer.[16] In particular, resveratrol showed cancer chemopreventive activity in assays

Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PDB, protein data bank; NMR, nuclear magnetic resonance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HEK, human embryonic kidney; PDS, 4,4′-dipyridyl disulfide; SAPD, sapintoxin-D; MALDI-TOF, matrix assisted light desorption ionization time of flight; ES-MS, electrospray mass spectrometry; COX, cyclooxygenase; LOX, lipooxygenase; p56lck, p65 lymphocyte-specific protein tyrosine kinase; ERK1, extracellular signal-regulated kinase; JNK1, c-Jun N-terminal protein kinase 1; IKKB, inhibitor of nuclear factor kappa B kinase beta; Src, sarcoma proto oncogene tyrosine kinase; Stat3, signal transducer and activators of transcription; PKD, protein kinase D; NQOR, NADH quinone oxidoreductase; NF-κB, nuclear factor κB; Sir2, silent information regulator 2; SIRT1, sirtuin-1; AMPK, AMP activated protein kinase; EGRF, epidermal growth factor receptor; Nrf2, NF-E2 related factor 2; PS, phosphatidylserine; *c* log *P*, logarithm of the calculated partition coefficient for *n*-octanol/water; BSA, bovine serum albumin.

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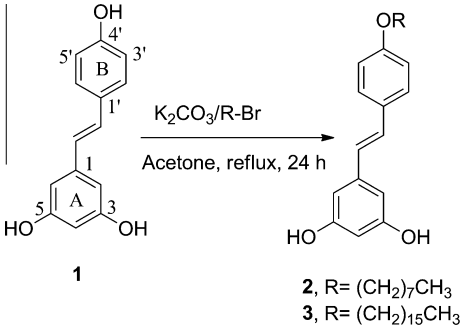

Scheme 1.

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representing anti-initiation, anti-promotion and anti-progression activity, inhibiting the development of preneoplastic lesions and tumorigenesis.[16] Further evidence showed that it inhibits cell growth and induces apoptosis in various human cancer cell lines.[17–21] In particular, a number of studies have been reported on the chemopreventive activity of resveratrol against prostate cancer.[22] More specifically, it showed differential effects on growth, cell cycle arrest, and induction of apoptosis in human prostate cancer cell lines.[23,24] Resveratrol is also currently in clinical phase II trials as an anti-cancer drug for treatment of human colon cancer.[25,26]

Resveratrol displays its biological response by acting on multiple targets. The activity of resveratrol has been linked to cell-surface receptors, membrane signaling pathways, intracellular signal-transduction machinery, nuclear receptors, gene transcription, and metabolic pathways.[27,28] Studies have shown that there is high degree of diversity in terms of the signaling networks and cellular effector mechanisms that are affected by resveratrol. Some of the known targets are, COX and LOX, p56lck, ERK1, JNK1, p38, IKKB, Src, Stat3, ribonucleotide reductase, DNA polymerases, PKD, NQOR, aromatase, NF-kB, Sir2/SIRT1, adenylate cyclase, AMPK and protein kinase C alpha (PKCα).

Protein kinase C alpha (PKCα)[29–31] belongs to the family of serine/threonine kinases involved in the regulation of various aspects of cell functions, including cell growth, differentiation, metabolism, and apoptosis.[32] PKC's role has been implicated in the pathology of several diseases such as cancer, diabetes, stroke, heart failure, and Alzheimer's disease.[33–39] PKC has been a subject of intensive research and drug development in the area of cancer.[40]

The PKC family has been divided into three main groups: conventional isoforms (α, βI, βII and γ) that require Ca^{2+} and diacylglycerol (DAG) for activation; novel isoforms (δ, ε, η, θ and μ) that require only DAG and atypical isoforms (ζ, ι and λ) that require neither Ca^{2+} nor DAG.[41] The conventional and novel PKCs have four domains, termed C1 through C4, that play distinct roles in kinases' function. C1 and C2 are regulatory domains, C3 is the ATP binding domain, and C4 is the catalytic domain. DAG contains two long chains and acts as a second messenger[42] by binding to the C1 domain and inducing the translocation of PKCs to discrete subcellular compartments. In the classical and novel PKC isoenzymes, the DAG-sensitive C1 domain is duplicated into a tandem C1 domain consisting of C1A and C1B subdomains. Along with the PKC family, there are six additional families of proteins that contain a DAG-responsive C1 domain.[43,44] The C1 domains have become an attractive target in designing the PKC based drugs. Recently it has been found that alcohol and anesthetics also bind to the PKC C1 domains.[45–47]

The biological effects of resveratrol on PKCα have been studied both in the cellular system and in in vitro purified proteins. Resveratrol regulates cellular PKCα and PKCδ to inhibit growth and induce apoptosis in gastric cancer cells.[48] It also inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells[49] and antagonizes EGFR-dependent Erk1/2 activation in human androgen-independent prostate cancer cells with associated isozyme-selective PKCα inhibition.[50] Resveratrol also preferentially inhibits PKC-catalyzed phosphorylation of a cofactor-independent, arginine-rich protein substrate by a novel mechanism.[51]

The mechanism of the effects of resveratrol on the activities of purified recombinant PKC isozymes induced by association with model lipid vesicle membranes was investigated using an in vitro assay system in which the cofactor and activator-concentration dependencies for activation were systematically varied.[52] It was found that resveratrol inhibited membrane-associated PKCα activity within a concentration range relevant to the cellular effects of the stilbene[11,16,53–55] and it was proposed that resveratrol binds to the C1 domain of PKCα.[52]

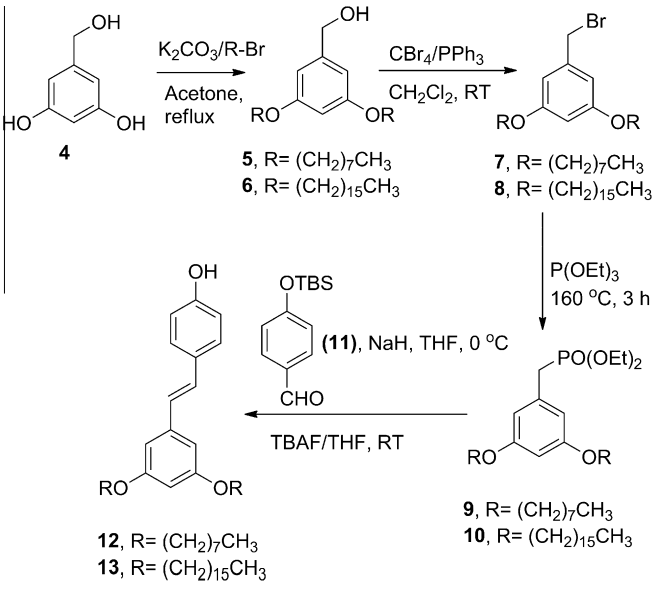
In the present study, we describe design, synthesis of several resveratrol derivatives and their effects on the translocation properties of PKCα in the presence and absence of a phorbol ester, TPA. The binding properties of resveratrol and its derivatives to the activator-binding C1 domain of PKCα were also studied using fluorescence spectroscopy and molecular docking. Our results show that derivatization of the hydroxyl groups of resveratrol with aliphatic carbon chain improved the PKCα activity. On the other hand it reduces the phorbol ester mediated PKCα inhibition. The binding and molecular docking studies indicate that resveratrol and its derivatives modulate PKCα activity by binding to its C1B domain.

2. Results

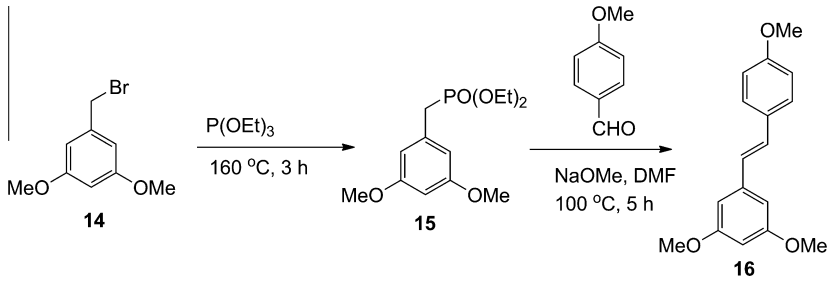
2.1. Chemical synthesis

In order to synthesize the monoalkoxy (at 4′ hydroxy) and dialkoxy derivatives (at 3 and 5 hydroxy) of resveratrol, commercially available (*E*)-resveratrol (**1**, Scheme 1) was first treated with alkyl bromide in the presence of K_2CO_3/acetone under refluxing condition. This reaction yielded the monoalkoxy derivatives of the 4′-OH in ring B of (*E*)-resveratrols (**2** and **3**) in moderate yield. The reaction also yielded dialkoxy and trialkoxy products in low yield. The dialkoxy and the trialkoxy products showed close retention time values, making it difficult to isolate them from the reaction mixture.

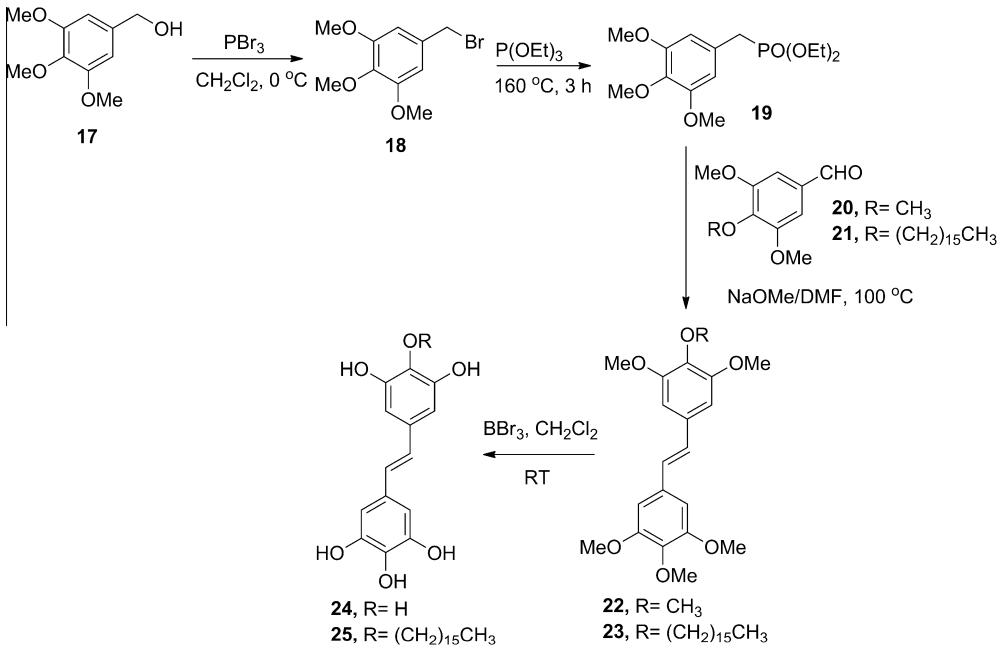
To prepare the dialkyl derivatives of 3 and 5 hydroxyl groups in resveratrol in good yield, we used the Wittig-Horner olefination that provided a convenient route (Scheme 2). The 3,5 dihydroxy substituted benzyl bromides (**7** and **8**) were treated with triethyl phosphite at 160 °C. The phosphonates generated in the Arbuzov rearrangement was used for the next step without further purification. The addition of the substituted benzaldehyde **11**, in the presence of NaH/THF at 0 °C led to resveratrol derivatives **12** and **13**, exclusively in the *E*-isomeric form. The phosphonium bromide of 3,5-dihydroxy substituted benzyl bromides (**7** and **8**) yielded both *Z*- and *E*-isomer in presence of *n*-BuLi at -78 °C. Trimethoxy resveratrol (**16**) was prepared in 95% yield by the reaction of diethyl 3,5-dimethoxybenzyl phosphonate (**15**) and 4-methoxy benzaldehyde (Scheme 3). Toward increasing the number of hydroxyl groups in resveratrol, we synthesized several compounds (**22–25**) using



Scheme 2.



Scheme 3.



Scheme 4.

methods outlined in Scheme 4. Compound **24** was prepared from **22** by treating with boron tribromide (BBr_3) which cleaved all the methyl groups. Synthesis of **25** using BBr_3 was more challenging and was carefully optimized to prevent the cleavage of the long chain alkoxy group. This compound was designed to study the effect of long chain and the increasing number of hydroxyl in the activation of PKCα. Some of the synthetic steps used in here were described earlier for the synthesis of variety of stilbene derivatives.[56–59]

2.2. Absorption and emission characteristics of resveratrol and its derivatives

Resveratrol (**1**) shows a broad absorption maximum in the range of 304–318 nm. This band does not show any significant solvent sensitivity when the solvent is switched from polar ethanol to nonpolar hexane (Table 1). For some of the derivatives such as **12** and **16**, two peaks were observed instead of a broad peak. Alkylation of one hydroxyl group as in **2** and **3**, and two hydroxyl groups

Table 1
Absorption and emission properties[a] of resveratrol and its derivatives in different solvents at 25 °C

Compound	Absorbance maximum (λ_{max}) (nm)				Emission maximum (λ_{em}) (nm)			
	EtOH	CH_3CN	Hexane	Water	EtOH	CH_3CN	Hexane	Water
1	306	305	306	310	379	377	373	392
2	308	312	306	304	377	374	371	363
								382
3	306	307	306	275	379	378	368	386
		319						
12	307	306	305	306	379	378	367	382
	319							
13	307	306	306	307	376	374	369	383
16	306	306	304	315	374	373	350	363
	317	318					368	381
22	325	326	328	326	386	391	385	393
24	331	328	324	325	394	385	380	406
25	330	328	328	326	395	384	383	405

[a] Concentration used was 1–5 × 10^{-6} M. For recording the emission spectra, excitation was done at the corresponding absorption maximum of each compound.

as in **12** and **13** or all the three hydroxyl groups as in **16** did not show any significant changes in the absorption maxima. On the other hand, the derivative **24** with six hydroxyl groups showed an absorption band in the range of 325–331 nm. For this derivative also, solvent effects was not significant. Again, when all the six hydroxyl groups were methylated in **22** or one of the hydroxyl was alkylated as in **25**, no shift in absorption maximum was observed. Absorption spectra of several resveratrol derivatives in ethanol are shown in Figure 1A.

The emission maximum of resveratrol is in the range of 373–379 nm in organic solvents, whereas it is red shifted to 392 nm in water. For the derivatives, the effect of solvent polarity on the wavelength shift does not follow any particular order. For all the compounds tested here, the emission maxima were blue shifted in the non polar solvent such as hexane as compared with ethanol or acetonitrile (Table 1). And, all the compounds show highest emission maxima in water. For compounds **2** and **16**, two distinct peaks were observed in water. Emission spectra of several resveratrol derivatives in ethanol are shown in Figure 1B.

2.3. Evaluation of cell viability of resveratrol derivatives

In earlier studies, resveratrol (5–200 μM) was shown to have cytotoxic effects in various cancer and non cancer cell lines.[60,61] In the present study, we treated HEK293 cells, in which we expressed PKCα in the subsequent experiments, with resveratrol and its derivatives (100 μM) for 48 h and the cell viability was measured by MTT assay. The results are shown in Figure 2. Cells treated with resveratrol (**1**) showed four- to fivefold decrease in cell viability (~20–25%) as compared to the untreated control cells.

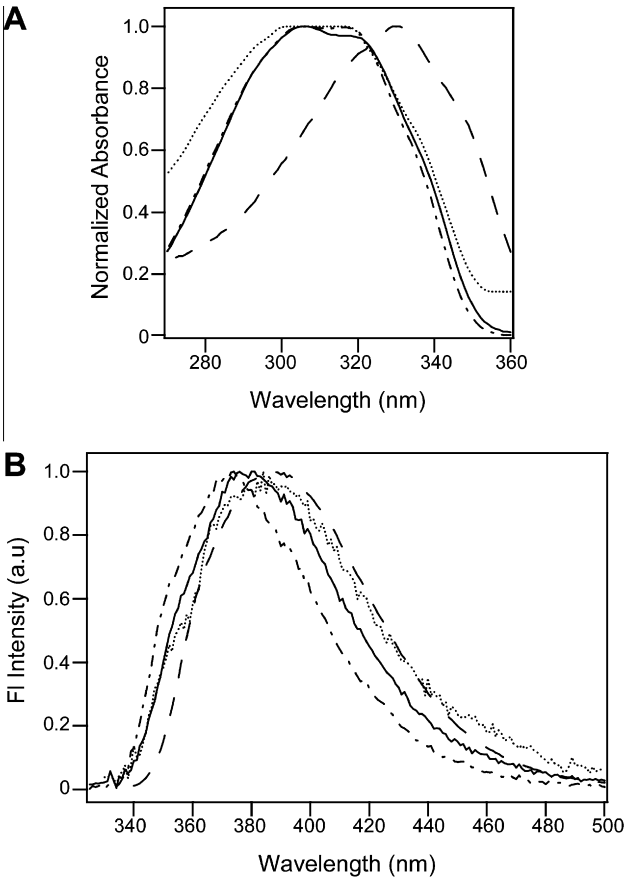


Figure 1. (A) Normalized absorption spectra and (B) normalized fluorescence emission spectra of **1** (—), **2** (···), **16** (– – – – –) and **24** (– – –) in ethanol.

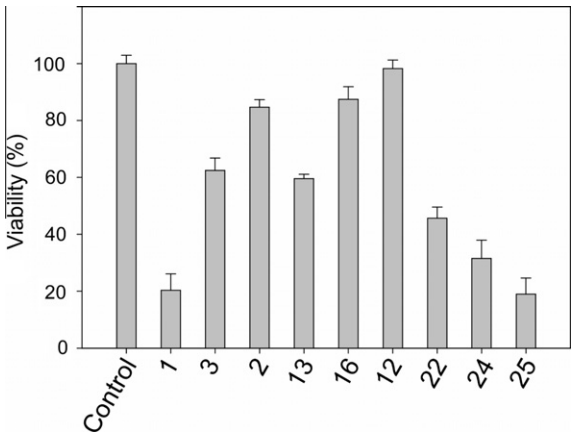


Figure 2. Effect of resveratrol and its derivatives on the cell viability. The bar graph shows the percent of viable HEK293 cells after treatment of resveratrol and its derivatives (100 μM) for 48 h as determined by MTT assay. Each bar represents the mean of 6 wells done in triplicate.

24 and **25**, the hydroxylated resveratrol analogues exhibited slightly higher cell viability (~25–30%) to that of resveratrol. Moreover, **16** and **22**, the methylated derivatives showed four- and twofold increase in cell viability respectively, as compared to resveratrol. Whereas four- to fivefold higher cell viability was observed for **2** and **12**, twofold higher cell viability was observed for **3** and **13**, as compared to resveratrol. These data indicate that methyl and long chain substitution of resveratrol significantly increase cell viability and reduced cytotoxicity as compared to resveratrol or its hydroxyl derivatives.

After measuring the HEK293 cell viability in the presence of resveratrol and its derivatives, we expressed PKCα in these cells and studied the effects of the derivatives in the TPA-induced membrane translocation, which is a measure of PKC activity.

2.4. Effects of resveratrol derivatives on TPA-induced PKCα membrane translocation

TPA is a high affinity and potent PKCα activator that causes its membrane translocation from cytoplasm to membrane.[62] Resveratrol was earlier shown to act as an inhibitor of TPA-induced PKCα membrane translocation.[50] Therefore, we decided to study the effects of resveratrol and its derivatives on TPA-induced PKCα membrane translocation in HEK293 cells transiently transfected with PKCα-EGFP. Figure 3 shows relative inhibition of TPA-induced PKCα membrane translocation by the resveratrol and its derivatives as detected by Western blot, after sub cellular fractionation. The data indicate that treatment of TPA (100 nM) alone increased the PKCα in membrane fraction (>90%). However, 20–30% of membrane PKCα was determined in the presence of resveratrol (100 μM, 2 h) and TPA (100 nM), which was significantly less (60–70%) as compared to TPA alone. Both these observations are in agreement with the results reported earlier.[50] However, varying amount of inhibition was observed for simultaneous treatment of resveratrol derivatives (100 μM) and TPA (100 nM). There were about 31.9%, 44.8%, 29.8%, 68.3%, 79.8%, 78.8%, 85.2% and 65.7% of the protein found in the membrane fraction for **16**, **22**, **24**, **25**, **3**, **13**, **2**, and **12**, respectively. Presence of one C_{16} chain in **3** or two chains in **13** did not show much difference in inhibition as compared to one another, but both showed 5–6 times reduction in TPA-induced inhibition as compared to resveratrol. No significant difference in inhibition was observed for compounds with a C_8 chain (in **2**) or a C_{16} chain (in **3**). However, addition of a second C_8 chain in **12** made this derivative more potent inhibitor (10%) as compared to **2**. Methylated derivatives (**16** and **22**) showed 10–20%

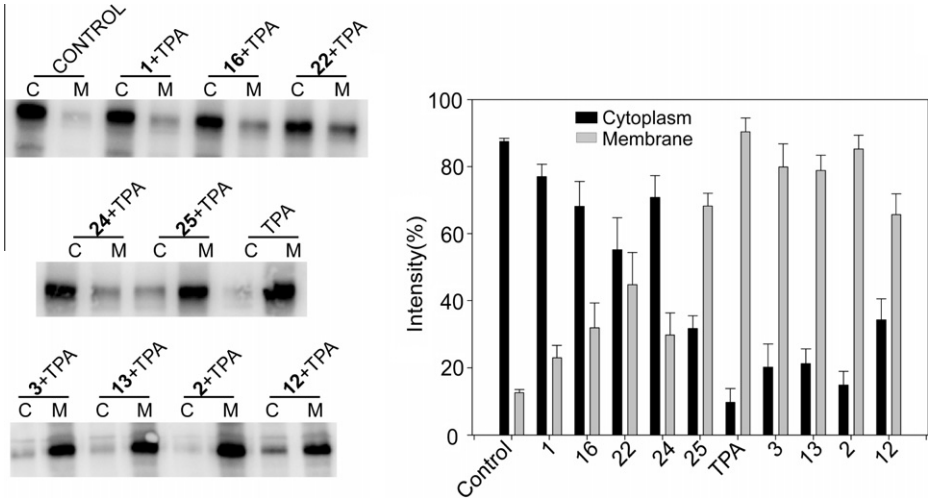


Figure 3. Effect of resveratrol and its derivatives on the TPA-induced membrane translocation of PKCα. Left, the Western blot analysis of PKCα in the cytosolic and membrane extract. HEK293 cells were transiently transfected with EGFP-PKCα and co-treated with resveratrol and its derivatives (100 μM) and TPA (100 nM) for 2 h. After cell fractionation, PKCα in cytosolic and membrane fraction was detected by Western blotting using anti-GFP antibody. Right, the corresponding densitometry analysis of Western blot is shown in the panel. Results are representative of three independent experiments.

less inhibition as compared to resveratrol. Similarly, 10–15% reduction in inhibition was observed when the number of hydroxyl group was increased to six in **24** as compared to resveratrol. However, addition of a C_{16} carbon chain to the **24** derivative (**25**) showed twofold reductions in inhibition. In summary, differential inhibitory effect of resveratrol derivatives on PKCα translocation was observed and resveratrol derivatives were found to be less potent inhibitor of TPA-induced PKCα translocation than resveratrol.

To investigate if these derivatives were able affect PKCα activity by themselves, we measured the PKCα membrane translocation in the absence of TPA, under similar experimental conditions.

2.5. Effects of resveratrol derivatives on basal PKCα membrane translocation

HEK293 cells transiently transfected with PKCαEGFP were treated with resveratrol derivatives (100 μM) for 2 h and PKC translocation was measured. Figure 4 shows the Western blot analysis of membrane and cytosolic factions after treatment with the resveratrol derivatives. Resveratrol inhibited or suppressed (~5%) basal PKCα membrane translocation as reported earlier.[50,51] Treatment with resveratrol derivatives significantly (20–25%) elevated the amount of PKCα in the membrane fraction as compared to either the untreated and resveratrol-treated cells. Among all the derivatives **16**, **22**, **3** and **25** showed higher extent of PKCα translocation to the membrane. Although these increments in activation are much lower than TPA, these increments are significantly higher as compared to resveratrol.

To investigate if resveratrol and its derivative regulate the PKCα activity by binding to the activator binding site, we purified, characterized and studied its binding with the resveratrol and its derivatives.

2.6. Purification and characterization of PKCαC1

Although the expression and purification of the PKCαC1 as a his_6-tagged protein has been described earlier[63], characterization was not done adequately. Because of the presence of 12 cysteine

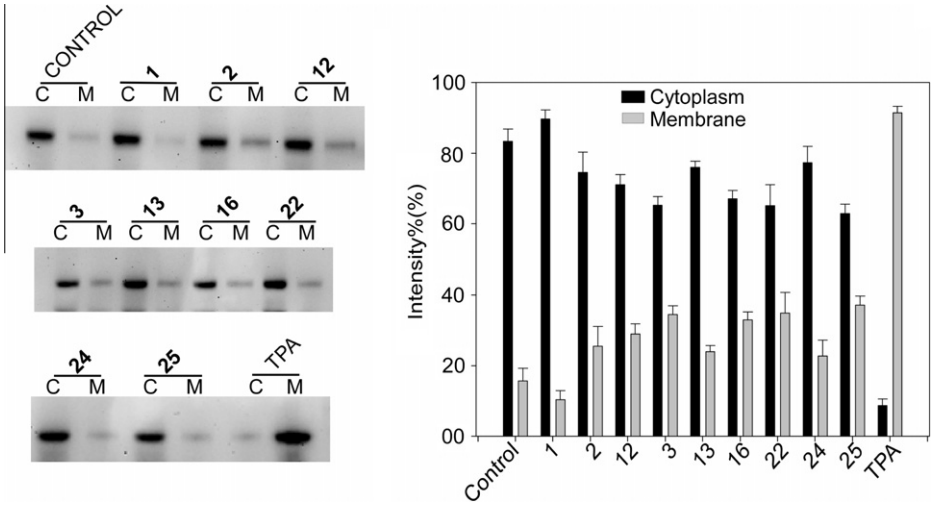


Figure 4. Effect of resveratrol and its derivatives on the membrane translocation of PKCα in the absence of TPA. Left, Western blot analysis of PKCα in the cytosolic and membrane extract. HEK293 cells were transiently transfected with PKCα and treated with resveratrol and its derivatives (100 μM) for 2 h. After the cell fractionation, PKCα was detected in cytosolic and membrane fraction by Western blotting using anti-GFP antibody. Right, the corresponding densitometry analysis of Western blot is shown in the panel. Results are representative of three independent experiments.

residues in this domain and the reported intrinsic instability and insolubility associated with this domain, we decided to characterize the protein thoroughly before using it for the binding studies with the resveratrol and its analogs. The characterization was done by several methods. The purity of protein was analyzed by SDS–PAGE (Fig. 5A) and the molecular mass was confirmed by mass spectrometric analysis (Fig. 5B), which showed the main peak is at around 14,234 Da as expected. Quantitation of water exposed cysteine residues with thiol reactive agent PDS showed that while in the denatured protein about 10 cysteines residues reacted with the PDS, no cysteine was reactive in the renatured protein indicating the folded state of this domain (Fig. 5C). In addition to this, the fluorescence emission maximum of PKCαC1 was at 336 nm, which is typical of a buried tryptophan residue. The functionality of the protein was tested by its binding to a fluorescent phorbol ester sapintoxin-D (SAPD). SAPD showed emission maximum at 438 nm in buffer, which is blue shifted to 428 nm in the presence of PKCαC1, indicating its binding to the protein (Fig. 5D).

2.7. Binding of resveratrol derivatives to PKCα C1

Resveratrol (**1**) and three of its derivatives **3, 16** and **24**, which showed higher activation in the membrane translocation assay, were selected for their binding to the PKCαC1. Figure 6A and B shows the plot of fluorescence quenching data for PKCαC1 in the presence of varying concentrations of the compounds. The EC_{50} values determined by fitting the fluorescence data using a modified Hill equation[45] are shown in Table 2. Resveratrol binds to the PKCαC1 with EC_{50} value of 5.8 μM with nH value of 1.14. Compounds **3**, **16**, and **24** bind to PKCα C1 with EC_{50} of 5.04 μM, 2.98 μM and 1.59 μM with nH values of 1.19, 1.17 and 1.67, respectively. The binding affinity was slightly higher for the long chain (**3**) derivative as compared to resveratrol. When the number of hydroxyl groups was increased, the binding affinity was also found to be increased in **24** as compared to resveratrol. The EC_{50} of the high affinity phorbol ester TPA was found to be 1.7 μM, suggesting that the phorbol ester and the resveratrol derivatives bind to the protein in a similar manner.

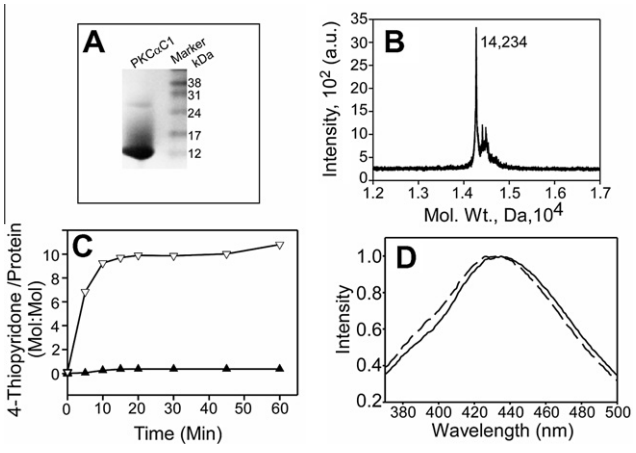


Figure 5. Characterization of PKCαC1. (A) SDS–PAGE (15%) of purified PKCαC1 stained with Coomassie blue. (B) Mass spectrometry analysis of purified recombinant PKCαC1. The observed mass of 14234 Da is close to the value of the calculated monoisotopic mass of 14242 Da. (C) Reaction of PDS with denatured (△) and refolded (▲) PKCαC1. The degree of labeling was presented as the ratio of moles of 4-thiopyridine released to the moles of protein used and plotted as function of time by measuring the absorbance of 4-thiopyridine at 323 nm, as described in the methods section. (D) Binding of SAPD with PKCαC1. Emission spectra of SPAD (1 μM) in the absence (—) and presence (– – –) of PKCαC1 (10 μM). Excitation was done at 355 nm. All spectra were background corrected.

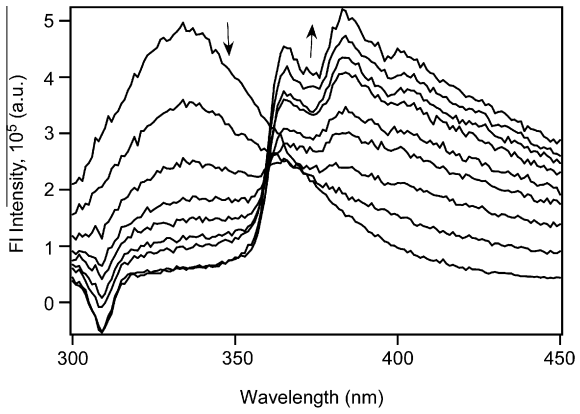


Figure 6A. Quenching of PKCαC1 fluorescence by resveratrol (**1**). Addition of increased concentration of resveratrol (0–32 μM) to PKCαC1 (2 μM) with an increment of 4 μM each time quenched the intrinsic florescence intensity at 336 nm and increased resveratrol fluorescence intensity at 380 nm, indicating the existence of energy transfer mechanism between the protein tryptophan and resveratrol. Excitation was at 280 nm.

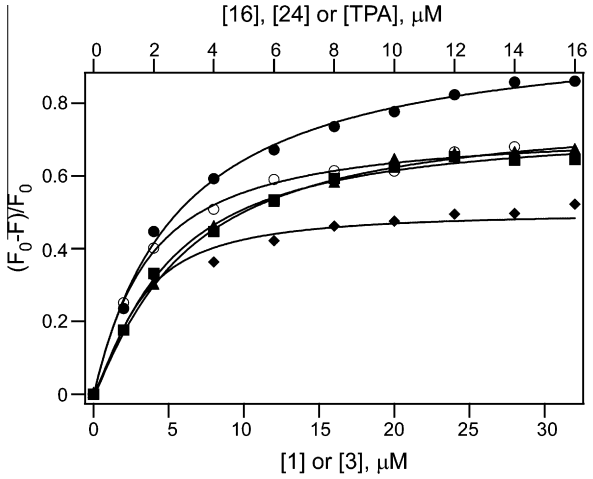


Figure 6B. Binding of resveratrol (**1**) and its derivatives with PKCαC1. Plot of fluorescence intensity of PKCαC1 (2 μM) in buffer (50 mM Tris, 150 mM NaCl, 2 mM DTT, 50 μM $ZnSO_4$, pH 7.2) in the presence of varying concentration of **1** (●), **3** (■), **16** (▲), **24** (♦) and TPA (○), where F and F_0 are fluorescence intensities in the presence and absence of the ligand, respectively. Solid lines indicate the fit using modified Hill equation; $f(x) = \text{Min} + \text{Max} - \text{Min}) \times x^{nH}/(x^{nH} + EC_{50}^{nH})$. The corresponding EC_{50} for **1**, **3**, **16**, **24** and TPA are 5.78, 5.04, 1.56, 2.98 and 1.70 μM, respectively, and the corresponding Hill coefficients (nH) are 0.99, 1.19, 1.67, 1.17 and 1.10, respectively. Excitation wavelength used was 280 nm.

Table 2
EC_{50} values and the corresponding Hill coefficients (nH) for the quenching of the intrinsic fluorescence of PKCαC1 by the resveratrol (**1**), its derivatives and TPA

Compounds	PKCαC1	
	EC_{50} (mM)	nH
1	5.78 ± 0.87	0.99 ± 0.11
3	5.04 ± 0.49	1.19 ± 0.12
16	1.56 ± 0.18	1.67 ± 0.39
24	2.98 ± 0.30	1.17 ± 0.15
TPA	1.7 ± 0.16	1.1 ± 0.13

PKCαC1 domain consists of two cysteine rich subdomains, C1A and C1B, the latter being the high affinity phorbol ester binding site. The fluorescence quenching observed in this experiment is mainly due to quenching of the intrinsic fluorescence arising from

Trp-58 of the C1A domain. This is the only tryptophan residue present in the entire C1 domain. The decrease of the tryptophan fluorescence at ~336 nm and the increase in the resveratrol fluorescence at ~395 nm with the concomitant addition of resveratrol (Fig. 6A) to the protein solution indicated that the energy transfer mechanism between the donor (tryptophan) and acceptor (resveratrol) was occurring. Similar type of energy transfer mechanism was observed for BSA-resveratrol and heamoglobin-resveratrol[64,65] interactions, which suggested that the distance between the donor and the acceptor was within the Foster's distance.[66]

To investigate if these compounds could interact with the high affinity phobol ester-binding site of the C1 domain, we studied their interaction with the C1B subdomain.

2.8. Binding of resveratrol derivatives to PKCα C1B

The binding of **1**, **3**, **16** and **24** was studied with the C1B domain, which is known to have higher affinity for phorbol ester TPA as compared to the C1A domain.[67] Because of absence of any tryptophan residue in this subdomain we studied the interactions of these compounds by measuring the change of their fluorescence emission maxima and intensity in the presence and absence of PKCαC1B. With the addition of protein, the fluorescence intensity of resveratrol increased and the emission maximum was 10 nm blue shifted as compared to its emission maximum in buffer. In addition to this blue shift, the broad emission band was split into two peaks at 365 and 383 nm (Fig. 7). These observations confirm that resveratrol binds to the C1B subdomain of PKCα. Similar observations were made for **16**, where the fluorescence intensity increased with the addition of protein and the broad peak was split into two peaks at 363 and 381 nm in the presence of protein. With further addition of protein, the intensity of the 363 nm peak became higher in intensity as compared to the 381 nm peak. For **3**, with the addition of the protein the peak intensity was increased, but the blue shift was less as compared to resveratrol. In contrast, a different observation was made for **24**. Addition of the protein to the **24** drastically quenched its fluorescence instead of the increase observed for the other derivatives, indicating a different type of interaction.

3. Discussion

The objective of the present study was to chemically modify resveratrol for improved PKCα binding and activity. Among of all the PKC subtypes, we selected PKCα for our study because there is compelling evidence in the literature that resveratrol modulates PKCα activity both in in vivo[50,51] and in vitro[52] systems. In addition, several other studies also revealed that resveratrol affected intersignalling pathways involving PKCα.[48,49,68] However there are no prior studies that systematically design molecules around the resveratrol scaffold for modulating PKCα activity.

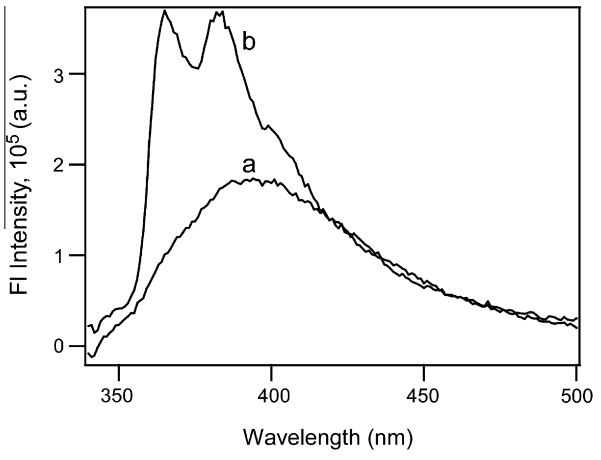


Figure 7. Emission spectra showing blue shift in emission maximum of resveratrol (5×10^{-6} M) in the absence (a) and presence (b) of PKCα C1B (20×10^{-6} M). Buffer used was 50 mM Tris, 100 mM NaCl, pH 7.2. Excitation was at 305 nm.

Naturally occurring phorbol esters, bryostatins, teleocidins, aplysiatoxins, ingenols, and iridals are known to bind and modulate PKC activity.[69–71] However, most of these compounds are highly complex in their chemical structure. Therefore, structural modification with the aim of altering the ligand specificity is difficult. In this study, we sought to find simpler template for ligand synthesis, whose structure could be easily modified and fine-tuned in order to achieve selectivity and should also be non-toxic. Using the same concept, very recently, isophthalic acid derivatives have been developed and tested for their binding to the activator binding domain of PKC and modulation of PKC activity.[72] In our previous study[73] also, we showed that another dietary polyphenol, curcumin and its derivatives bind to the C1B subdomains of novel protein kinase Cs by forming hydrogen bonds with the residues at the activator binding site.

Here we synthesized several new resveratrol analogs and studied their effect on the membrane translocation properties of PKCα in the presence and absence of a PKC activator, TPA and studied their binding with the activator binding domain of PKCα. The analogs were rationally designed to study the effect of (1) increasing number of hydroxyl group, (2) alkylation of hydroxyl groups and (3) addition of long chain to the specific hydroxyl group, to mimic the long chains of the PKC activator diacylglycerol and TPA. The importance of hydroxy pharmacophore in phorbol ester in binding to the protein has been highlighted in the PKCδC1B-phorbol-13-OAc crystal structure[74] and significance of the hydrophobic long chain of DAG and TPA in membrane binding and PKC activation have been elucidated earlier.[75,76]

Our results indicate that the cytotoxic effects of the resveratrol analogs are less as compared to the parent resveratrol, when tested in HEK293 cells. In the absence of TPA, most of these analogs show activation, which is higher than resveratrol, but lower than that of TPA. In the presence of TPA however, the analogs showed reduced PKCα inhibition as compared to resveratrol. The activity was measured from the extent of translocation of the over expressed PKCα from cytoplasm to the membrane in HEK293 cells. Furthermore, we show that the resveratrol derivatives bind to the C1B domain of PKCα.

In the DAG-induced activation of PKCα, its membrane binding is initiated by the Ca^{2+}-dependent adsorption of the C2 domain to the anionic membrane surface. Binding of a phosphatidylserine (PS) molecule in the membrane to the Ca^{2+}-binding loops of the C2 domain would cause a local conformational change that would result in unleashing of the C1A domain, which then penetrates the membrane and binds DAG. This movement of the C1A domain would not only enhance the membrane-binding energy but also remove the pseudosubstrate from the active site, leading to enzyme activation.[77,78] In contrast to DAG, the phorbol ester mediated membrane translocation and activation mechanism for PKCα is somewhat different given the opposite binding affinity of its C1A and C1B domains for DAG and phorbol ester.[78,79] This difference in binding and activation was highlighted earlier,[74,75,80] which suggested that phorbol ester induced an irreversible insertion of the protein into the membrane and produced kinase activity, which is calcium independent. The kinase activity was however depended on the lipophilicity of the phorbol esters and the phosphotidylserine concentration.[75,76] In addition to this, Bazzi and Nelsestuen described the ability of PKC to form another membrane-associated state, which is calcium-dependent and reversible upon calcium chela-

tion.[80] The high population of the hydrophobic residues in the phorbol ester-binding site favors the membrane insertion, which could be about 6–8 Å. The binding of phorbol ester in the activator-binding groove satisfies the hydrogen bonds, which makes the membrane insertion energetically favorable.[74]

The activation observed in the case of resveratrol analogs in the absence of TPA, is presumably due to their interaction with the C1 domain. The different extent of activation or membrane translocation for these analogs is due to their different lipophilicity. The higher activation observed for **2** (*c* log *P* 7.12), **12** (*c* log *P* 11.5) and **25** (*c* log *P* 9.15) as compared to resveratrol (*c* log *P* 2.833) correlates well with the lipophilicity and activation characteristics. The higher activation shown by the long chain derivatives may be the result of its stronger interaction with the full-length protein and the membrane lipid. The smaller extent of inhibition observed for resveratrol is probably due to its particular orientation and interaction, which could be similar to the case of 4α-TPA that has just one inverted stereo center[81,82] relative to TPA and does not activate PKC at all.

The inhibition of PKCα by resveratrol has earlier been proposed due to its competition for the phorbol ester binding site.[52] The EC_{50} values for the binding of resveratrol, its derivatives and TPA indicate that these analogs bind the C1 domain in a similar manner suggesting for the presence of a common binding site.

Between the C1A and C1B subdomains, we showed that the resveratrol and its analogs bind to the C1B subdomain, which is also a high affinity site for phorbol esters.[67,79] Binding of both phorbol ester and resveratrol derivatives in this subdomain thus explains both activation in the absence of TPA and the inhibition of TPA-induced membrane translocation by these compounds. This however does not completely rule out the possibility of their binding to the C1A subdomain since phorbol ester also binds to the C1A subdomain, albeit with much lower affinity than C1B[67], and the observation of energy transfer involving Trp-58 of the C1A and resveratrol. This energy transfer indicates that either the resveratrol molecules are populated in the C1A domain or this domain orients itself in the PKCαC1 in such a way that the distance of the resveratrol molecules residing in the C1B domain are within the limit of Fosters energy transfer distance.[66] The recently published crystal structure of PKCβII, a conventional PKC, however, could not shed light on the orientation of C1A and C1B relative to each other, since the entire C1A subdomain was unobserved in this structure.[83]

The crystal structure[74] of PKCδC1B complexed with phorbol-13-OAc revealed that the hydroxyl groups attached to the C20 and C4 and the carbonyl group on C3 formed hydrogen bonds with protein residues indicating the role of hydroxyl groups in the activator protein interactions. Furthermore, when phorbol-13-OAc was docked into the PKCαC1B NMR structure template,[84,85] three out of the four hydrogen bonds were formed between the 9-OH and the Gly-124 and the fourth one was between 20-OH and Gln-128. Resveratrol with its three hydroxyl pharmacophores, is expected to bind to the C1B domain by forming hydrogen bonds with the protein residues. In fact, docking of resveratrol molecule into the PKCαC1B (Figure 8) resulted a total score value of 2.96 and four possible hydrogen bonds of which two were with 5-OH (with Thr-113 and Gln-128), one with the 3-OH (with Gly-110), and another with 4′-OH (with Gly-124), reaffirming our prediction of the role of hydroxyl groups in its binding to the C1B domain. Crystal structure of several resveratrol–protein complexes[86–89] also revealed that the hydroxyl group of resveratrol formed hydrogen bond with the protein residues either directly or mediated by water molecules.

Molecules that selectively target of PKCα, have potential therapeutic value in a wide variety of disease states, such as cardiac contractility, atherogenesis, cancer and arterial thrombosis. It has been postulated that the inhibitory effect of resveratrol on PKCα activity may partially underlie the mechanisms by which resveratrol exerts its beneficial effects on endothelial and cardiovascular function. However, in case of several types of cancer, both up regulation and down regulation of PKCα, depending on the type of cancer and the tissue, has been reported. In addition to the above mentioned disease states, these resveratrol derivatives may also find application in EGRF-dependent ERK1/2 activation, which is associated with the PKC signaling pathways[50] in cancer prevention. Additionally, resveratrol and its derivatives, by virtue of their different antioxidant properties[90], may also interact with the antioxidant enzymes such as Nrf2[91–93] differently, thereby producing varying biological response. However, it must be noted that complication of using these derivatives for a particular disease state may arise due to the opposing effect of one isoform to the other. For example, PKCα and PKCδ play opposite roles in the proliferation and apoptosis of glioma cells.[94] Other examples include PKCε and PKCγ in alcoholism[95] and PKCδ and PKCε in cardioprotection.[96] Therefore, broadly, the effects of resveratrol and its derivatives on the PKC signaling networks would likely to differ according to the cellular localization and types of PKC isoforms. This means that the studies on the effects of these derivatives on the modulation of other PKC isoforms are also warranted.

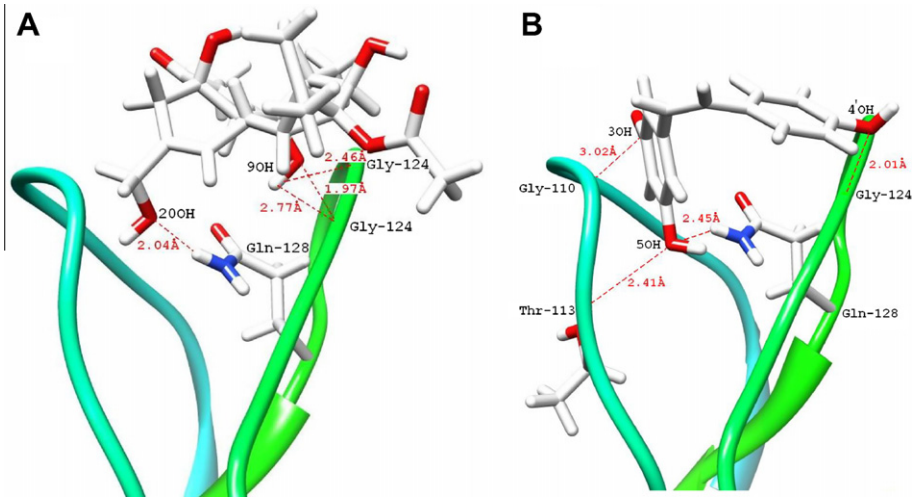


Figure 8. Docking of (A) TPA and (B) resveratrol (**1**) into PKCαC1B. The docked structures were generated using the Surflex dock module of Sybyl 8.0 and visualized using Chimera package from the Computer Graphics Laboratory, University of California San Francisco, San Francisco, CA, USA. The NMR structure of PKCαC1B (PDB code: 2ELI) was used as the receptor template.

In conclusion, we have synthesized several analogs of a dietary polyphenol resveratrol and characterized their PKCα binding and activation properties. The chemical modifications improved the cytotoxic effects and the activation properties of resveratrol, whereas TPA induced inhibition was reduced as compared to resveratrol. These results suggest that PKCα activity could be attenuated by chemical modification of resveratrol, which could be used as a strategy for regulating various disease states involving PKCα.

4. Experimental section

4.1. Materials and general procedures

TPA and sapintoxin-D (SAPD) were purchased from LC Laboratories, Woburn, MA. Solvents were purchased from VWR International, Radnor, PA and Fisher Scientific, Pittsburg, PA. All other reagents were from Aldrich and used without further purification. Progress of chemical reaction was monitored through thin layer chromatography (TLC) on pre-coated glass plates (Silica Gel 60 F254, 0.25 mm thickness) purchased from EMD chemicals. ^{1}H NMR and ^{13}C NMR spectra were recorded on a QE-300 spectrometer and JEOL-400 spectrometer. Unless otherwise specified, all NMR spectra were obtained in deuterated chloroform ($CDCl_3$) and referenced to the residual solvent peak; chemical shifts are reported in parts per million, and coupling constants in Hertz (Hz). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet) and br (broadened). Mass spectra were obtained on either a VG 70-S Nier Johnson or JEOL Mass spectrometer or Applied Biosciences Voyager System 4160. Absorption spectra were recorded on Hitachi U-2910 spectrophotometer. Sonications were done using Branson digital sonifier (model no 450). Fluorescence measurements were carried out using PTI fluorimeter PTI LPS 220B (Photon Technology Instruments, Princeton, NJ). *c* log *P* values of the compounds were calculated using ChemBioDraw ultra 12.0 (cambridgeSoft).

The PKCα-pEGFPN1 expression vector was kindly provided by Dr. P. M. Blumberg of National Cancer Institute, Bethesda. Anti-GFP and anti-rabbit IgG HRP antibody were from Cell Signaling Technology (Woburn, MA, USA). All cell culture reagents were from Invitrogen (Carlsbad, CA, USA).

4.1.1. Synthesis of (*E*)-5-(4-(octyloxy)styryl)benzene-1,3-diol (2) and (*E*)-5-(4-(hexadecyloxy)styryl)benzene-1,3-diol (3)

1-Bromooctane or 1-bromohexadecane (1.1 equiv) was added to a stirred solution of resveratrol (**1**) (368 mg, 1 equiv) and K_2CO_3 (1.1 equiv) in dry acetone (10 ml) and the mixture was refluxed for 24 h. After cooling the mixture to room temperature and filtering, the solvent was removed in vacuo. The resulting residue was subjected to column chromatography (*n*-hexane–EtOAc) to obtain **2** and **3**.

Compound **2:** Yield: 52%, ^{1}H NMR (CD_3OD) δ 7.50 (2H, d, J = 8.7 Hz), 7.04 (1H, d, J = 16.2 Hz), 6.94 (2H, d, J = 8.7 Hz), 6.89 (1H, d, J = 16.2 Hz), 6.51 (2H, d, J = 2.1 Hz), 6.22 (1H, t,, J = 2.1 Hz), 4.03 (2H, t), 1.82 (2H, q), 1.51–1.35 (10H, m), 0.96 (3H, t); ^{13}C NMR (CD_3OD) δ 158.8, 158.2 (2C), 139.7, 129.9, 127.6, 127.2 (2C), 126.2, 114.2 (2C), 104.3, 101.2, 67.5, 31.5, 29.0, 28.9, 25.7, 22.2, 12.9; ES-MS: 340 [M^+].

Compound **3:** Yield: 57%, ^{1}H NMR (CD_3OD) δ 7.49 (2H, d, J = 8.7 Hz), 7.04 (1H, d, J = 16.2 Hz), 6.96 (2H, d, J = 8.7 Hz), 6.89 (1H, d, J = 15.3 Hz), 6.51 (2H, d, J = 2.1 Hz), 6.22 (1H, t,, J = 2.1 Hz), 4.04 (2H, t), 1.83 (2H, q), 1.54–1.35 (26H, m), 0.96 (3H, t); ^{13}C NMR (CD_3OD) δ 158.8, 158.2, 139.7, 129.9, 127.6, 127.2, 126.1, 114.2, 104.37 (2C), 94.5, 67.5, 38.0, 31.6, 29.3 (2C), 29.2, 29.0, 28.9, 25.7, 22.3, 13.0. ES-MS: 476 [M+Na].

4.1.2. General procedure for preparation of (3,5-bis(octyloxy)phenyl)methanol (5) and (3,5-bis(hexadecyloxy)phenyl)methanol (6)

A mixture of 3,5-dihydroxybenzylalcohol (**4**) (1 equiv), alkyl bromide (1-bromooctane or 1-bromohexadecane) (2.2 equiv) and K_2CO_3 (2.2 equiv) in dry acetone was refluxed under argon for 24 h. The mixture was cooled to room temperature, followed by solvent removal under vacuum and addition of water. The aqueous layer was extracted with ethyl acetate and the organic layer was concentrated under reduced pressure to obtain the crude reaction mixture which was purified by silica gel column chromatography using ethyl acetate/hexane (25:75) as the eluent.

Compound **5**: Yield: 96%, ^{1}H NMR ($CDCl_3$) δ 6.58 (2H, d, J = 2.1 Hz), 6.45 (1H, t), 4.69 (2H, d, J = 6.0 Hz), 4.01 (4H, t, J = 6.6 Hz), 1.84 (4H, quin, 1.52–1.36 (20H, m), 0.96 (6H, t, J = 6.6 Hz).

Compound **6**: Yield: 89%, ^{1}H NMR ($CDCl_3$) δ 6.49 (2H, d, J = 2.1 Hz), 6.37 (1H, t), 4.61 (2H, d, J = 6.0 Hz), 3.93 (4H, t, J = 6.6 Hz), 1.76 (4H, quin), 1.42–1.25 (52H, m), 0.87 (6H, t, J = 6.3 Hz).

4.1.3. Synthesis of 1-(bromomethyl)-3,5-bis(octyloxy)benzene (7) and 1-(bromomethyl)-3,5-bis(hexadecyloxy)benzene (8)

To a stirring solution of appropriate benzyl alcohol **5** or **6** (1 equiv) and Ph_3P (1.2 equiv), CBr_4 (1.2 equiv) was added under argon. The reaction was monitored until completion by thin layer chromatography. If the reaction was not complete after 10 min, an additional 1 equivalent of Ph_3P and CBr_4 were added. Upon completion of the reaction, water was added to the reaction mixture. The aqueous layer was extracted with ethyl acetate and the organic layer was concentrated under reduced pressure to produce the crude reaction mixture. The crude product was purified by silica gel column chromatography using ethyl acetate/hexane (10:90) as the eluent.

Compound **7**: Yield: 95%, ^{1}H NMR ($CDCl_3$) δ 6.51 (2H, d, J = 2.1 Hz), 6.37 (1H, t), 4.39 (2H, s), 3.91 (4H, t, J = 6.3 Hz), 1.75 (4H, quin), 1.43–1.28 (20H, m), 0.88 (6H, t, J = 6.6 Hz).

Compound **8**: Yield: 91%, ^{1}H NMR ($CDCl_3$) δ 6.51 (2H, d, J = 2.4 Hz), 6.37 (1H, t), 4.40 (2H, s), 3.92 (4H, t, J = 6.6 Hz), 1.78 (4H, quin), 1.46–1.20 (52H, m), 0.86 (6H, t, J = 6.4 Hz).

4.1.4. Synthesis of diethyl 3,5-bis(octyloxy)benzylphosphonate (9) and diethyl 3,5-bis(hexadecyloxy)benzylphosphonate (10)

3,5-Dialkoxybenzyl bromide (**7** or **8**) was dissolved in excess triethyl phosphate and the solution was heated to 160 °C for 3 h. Excess triethyl phosphite was removed by heating at 80–90 °C under high vacuum and the remaining colorless phosphonate was used without further purification.

4.1.5. Synthesis of 4-((*tert*-butyldimethylsilyl)oxy) benzaldehyde (11)

4-Hydroxybenzaldehyde was dissolved in CH_2Cl_2 and cooled to 0 °C. To this was added *tert*-butyldimethylsilyl chloride (1.2 equiv) and imidazole (3 equiv). The mixture was allowed to warm to room temperature for 5 h. Water was added to quench the reaction and the mixture was extracted with two portions of CH_2Cl_2. The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure.

4.1.6. Synthesis of (*E*)-4-(3,5-bis(octyloxy)styryl)phenol (12) and (*E*)-4-(3,5-bis(hexadecyloxy)styryl)phenol (13)

The compound **9** or **10** was dissolved in dry THF (20 ml) and stirred at 0 °C. Sodium hydride (0.6 g, 25 mmol) was added to the well-stirred phosphonate ester solution. After 30 min, the aldehyde **11** (10 mmol) in THF (30 ml) was added dropwise, and the mixture was allowed to stir at room temperature for 8–16 h. The

mixture was then cooled to 0 °C, and the excess sodium hydride was quenched with water (10 ml). The reaction mixture was then poured on ice, followed by addition of 1 M HCl to pH 6, and the product was extracted with ethyl acetate (4 × 50 ml). The organic layers were combined and washed with saturated solution of sodium chloride (2 × 30 ml). The ethyl acetate layer was dried over anhydrous sodium sulfate and evaporated. The crude reaction mixture was purified by silica gel column chromatography using ethyl acetate/hexane (3:97) as the eluent.

Compound **12:** Yield: 64%, ^{1}H NMR (CD_3OD) δ 7.40 (2H, d, J = 9.0 Hz), 7.02 (1H, d, J = 16.5 Hz), 6.87 (1H, d, J = 16.5 Hz), 6.82 (2H, d, J = 8.7 Hz), 6.63 (2H, d, J = 2.1 Hz), 6.37 (1H, t, J = 2.1 Hz), 3.92 (2H, d, J = 6.6 Hz), 1.79 (4H, m), 1.46–1.26 (22H, m), 0.89 (6H, t); ^{13}C NMR (CD_3OD) δ 160.4, 155.2, 133.4, 130.2, 128.3, 127.9, 115.5, 104.85, 68.0, 31.8, 29.3, 29.2, 29.2, 26.0, 22.6, 14.1. ES-MS: 453 [M+ H]; HRMS calcd for $C_{30}H_{44}O_3$ [M + H]$^+$: 453.3348. Found: 453.3339.

Compound **13:** Yield: 60%, ^{1}H NMR (CD_3OD) δ 7.47 (2H, d, J = 9.0 Hz), 7.09 (1H, d, J = 16.5 Hz), 6.94 (1H, d, J = 15.7 Hz), 6.91 (2H, d, J = 8.7 Hz), 6.70 (2H, d, J = 2.1 Hz), 6.44 (1H, t), 4.04 (4H, t), 1.83 (4H, m), 1.51–1.25 (52H, m), 0.93 (6H, t); ^{13}C NMR (CD_3OD) δ 160.4, 155.2, 139.4, 130.2, 128.3, 127.9, 126.7, 115.5, 104.8, 68.0, 31.9, 29.6, 29.5, 29.4, 29.3, 29.2, 26.0, 22.6, 14.1. ES-MS: 677 [M+]; HRMS calcd for $C_{46}H_{76}O_3$: 677.5876. Found: 677.5845.

4.1.7. Synthesis of (*E*)-1,3-dimethoxy-5-(4-methoxystyryl)benzene (16)

3,5-Dimethoxybenzyl bromide (**14**) was dissolved in excess triethyl phosphite and the solution was heated to 160 °C for 3 h. Excess triethyl phosphite was removed by heating at 80–90 °C under high vacuum and the remaining colorless phosphonate was used without further purification. A solution of the phosphonate (**15**) in DMF was treated with 5 equiv of NaOMe and cooled to 0 °C prior to the addition of the corresponding 4-methoxybenzaldehyde. After the reaction mixture was heated to 100 °C for 5 h and the reaction quenched with methanol/water, the precipitated crystals were filtered off and washed with water. Chromatography (silica gel, toluene) afforded the pure product.

Compound **16**: Yield: 94%, ^{1}H NMR (CD_3OD) δ 7.54 (2H, d, J = 8.7 Hz), 7.15 (1H, d, J = 16.2 Hz), 7.05 (1H, d, J = 15.7 Hz), 6.98 (2H, d, J = 8.7 Hz), 6.76 (2H, d, J = 2.4 Hz), 6.486 (1H, t), 3.91 (6H, s), 3.89 (3H, s). ES-MS: 271 [M+H]; HRMS calcd for $C_{17}H_{18}O_3$ [M+H]$^+$: 271.1300. Found: 271.1290.

4.1.8. Synthesis of 5-(bromomethyl)-1,2,3-trimethoxybenzene (18)

PBr_3 (2 equiv) was added to a solution of the 3,4,5-trimethoxybenzyl alcohol (**17**) (1 equiv) in dry CH_2Cl_2 (2 ml), under argon at 0 °C. The mixture was stirred at room temperature and the reaction was monitored by TLC until completion. The resulting mixture was hydrolyzed with water (10 ml) and extracted with $CHCl_3$ (3 × 15 ml). The organic layer was washed with brine (2 × 10 ml), separated by decantation and dried over anhydrous $MgSO_4$. Solvent evaporation at reduced pressure provided the expected product which was utilized without any further purification.

4.1.9. Synthesis of diethyl (3,4,5-trimethoxybenzyl)phosphine oxide (19)

Synthesis of compound **19** from **18** was done following the same methods used for the synthesis of **9** or **10**.

4.1.10. Synthesis of 4-(hexadecyloxy)-3,5-dimethoxybenzaldehyde (21)

Bromohexadecane (1 equiv) was added to a stirred solution of 3,5-dimethoxy-4-hydroxybenzaldehyde (1 equiv) and NaH (1 equiv) in dry DMF (10 ml) followed by addition of a pinch of potassium iodide at 0 °C. After 30 min, the mixture was heated for overnight at 50 °C. The mixture was quenched with cold water and extracted with ethyl acetate (3 × 10 ml). The ethyl acetate layer was dried over anhydrous sodium sulfate and evaporated. The crude reaction mixture was purified by silica gel column chromatography using ethyl acetate/hexane (5:95) as the eluent.

Compound **21**: Yield: 87%, ^{1}H NMR ($CDCl_3$) δ 9.85 (1H, s), 7.11 (2H, s), 4.06 (2H, t), 3.90 (6H, s), 1.75 (2H, m), 1.42 (2H, m), 1.28–1.24 (24H, m), 0.86 (3H, t); ^{13}C NMR (CD_3OD) δ 190.2, 152.0, 144.2, 133.9, 105.6, 56.0, 73.5, 28.5, 23.2, 14.1. ES-MS: 407 [M+H].

4.1.11. Synthesis of (*E*)-1,2-bis(3,4,5-trimethoxyphenyl)ethene (22) and (*E*)-2-(hexadecyloxy)-1,3-dimethoxy-5-(3,4,5-trimethoxystyryl)benzene (23)

In a flame-dried three-necked flask the corresponding methoxylated diethyl benzylphosphonate (**19**, 1 equiv) was cooled to 0 °C under argon. Then 10 ml dry DMF, sodium methoxide (2 equiv) and the corresponding methoxylated benzaldehyde (**20** or **21**) (1 equiv) were added. The mixture was stirred at room temperature for 1 h, and then heated to 100 °C under argon for 3 h. The solution was poured into 250 ml ice-water and the precipitate was filtered off and recrystallized from diluted or pure ethanol to yield white crystals.

Compound **22**: Yield: 79%, ^{1}H NMR ($CDCl_3$) δ 6.92 (2H, s), 6.72 (4H, s), 3.90 (12H, s), 3.85 (6H, s). ES-MS: 360 [M+], 361 [M+H].

Compound **23:** Yield: 77%, ^{1}H NMR ($CDCl_3$) δ 6.93 (2H, s), 6.72 (4H, s), 3.97 (2H, t), 3.91 (6H, s), 3.89 (6H, s), 3.86 (3H, s), 1.75 (2H, m), 1.42 (2H, m), 1.28–1.24 (24H, m), 0.86 (3H, t); ^{13}C NMR (CD_3OD) δ 153.0, 138.4, 135.2, 129.2, 127.4, 103.9, 73.6, 60.5, 28.5, 23.2, 14.0. ES-MS: 570 [M+], 571 [M+H].

4.1.12. Synthesis of (*E*)-5,5′-(ethene-1,2-diyl)bis(benzene-1,2,3-triol) (24) and (*E*)-5-(3,5-dihydroxy-4-(octyloxy)styryl)benzene-1,2,3-triol (25)

In a two-necked flask the corresponding methoxystilbene (**22** or **23**) (1 equiv) was dissolved in dry methylene chloride under argon and cooled to −42 °C with dry ice/acetonitrile. Then boron tribromide (1 M solution in methylene chloride, 3 equiv per methoxy-group) was added via syringe. The solution was warmed to room temperature and stirred for 4 and 2 h, respectively, for **24** and **25**. The reaction was then quenched by slowly adding 20 ml water. After stirring for another 30 min the methylene chloride was evaporated and the water-phase was extracted three times with ethyl acetate. The organic layers were dried over Na_2SO_4 and the solvent was removed in vacuo. The resulting material was recrystallized from ethanol/water.

Compound **24:** Yield: 78%, ^{1}H NMR (CD_3OD) δ 6.62 (2H, s), 6.46 (4H, s); ^{13}C NMR (CD_3OD) δ 145.6, 132.6, 129.3, 126.0, 105.0.

Compound **25**: 4.1.13. Yield: 82%, ^{1}H NMR (CD_3OD) δ 6.62 (2H, s), 6.46 (4H, s), 3.41 (2H, t), 1.81 (2H, m), 1.41 (2H, m), 1.28–1.24 (24H, m), 0.88 (3H, t); ^{13}C NMR (CD_3OD) δ 152.0, 145.6, 132.9, 138.2,129.2, 126.0, 104.9, 72.7, 28.5, 23.2. MALDI: 523 (M+Na).

4.2. Expression and purification of PKCαC1 and PKCαC1B

The PKCαC1 was cloned in pET28a$^+$ (Novagen) with a carboxy terminal containing hexahistidine tag. The recombinant plasmid was transformed in *Escherichia coli* BL21 (DE3) Gold for expression and purification. Single colony from each freshly transformed plate was inoculated in 10 ml LB (Luria Broth) medium with ampicilin (100 μg/ml) and the culture was grown overnight at 37 °C at 225 rpm. Two liters of cultures (TB (Terrific Broth)–ampicilin) were inoculated with the 10 ml culture containing transformed *E. coli* and were allowed to grow at 37 °C until the OD_{600nm} reached to 0.4. Protein expression was induced with 0.5 mM IPTG, and cul-

tures were allowed to grow for an additional 4 h at 37 °C. The cells were harvested by centrifugation (6000*g* for 5 min) at 4 °C and resuspended in 20 ml of buffer A (50 mM Tris, pH 10, 100 mM NaCl, 50 μM $ZnSO_4$, 8 M Urea) and kept at room temperature for 1 h. The cells were then sonicated 20 times for 5 s at 30% amplitude and insoluble cellular debris was removed by centrifugation (17,000*g* for 30 min at 22 °C). The supernatant containing the denaturated protein fraction were passed through Ni–NTA agarose column (1 ml). Column was washed with buffer B (buffer A plus 10 mM imidazole) until maximum amounts of impurity were removed. Protein elution was carried out with elution buffer (buffer A containing 500 mM L-arginine, 10 mM cystein protease inhibitor and 300 mM imidazole). Refolding was initiated by dilution of the denatured protein suspension to upto 0.5 M urea concentration with refolding buffer (50 mM Tris, pH 10, 100 mM NaCl, 50 μM $ZnSO_4$, 250 mM L-arginine). Refolded protein was concentrated and dialyzed with the above refolding buffer. Molecular weight of purified protein was confirmed by MALDI-TOF mass spectrometry.

PKCαC1B was expressed in *E. coli* as a GST-tagged protein, purified by glutathione sepharose column and the GST tag was removed by the thrombin treatment using similar methods described earlier.[45–47]

4.3. Fluorescence measurements

A 1.5-ml cuvette (Hellma) with a Teflon stopper was used for fluorescence measurements. Binding of sapintoxin-D (SAPD) with PKCαC1 was carried out by recording the emission spectra of 1 μM SAPD in the presence (10 μM) and absence of PKCαC1. SAPD and protein were mixed in buffer (50 mM Tris, 100 mM NaCl and 50 μM $ZnSO_4$, pH 8) and stirred gently for 30 min before recording the spectra. SAPD was excited at 355 nm and emission spectra were recorded from 375 to 575 nm.

For fluorescence quenching experiments, PKCαC1 (1 μM) and varying concentration (0–30 μM) of resveratrol (**1**) and its derivatives (**3**, **16**, **24**) were incubated in a buffer solution (50 mM Tris, 150 mM NaCl, 50 μM $ZnSO_4$, pH 8) at 25 °C. Protein was excited at 280 nm and emission spectra were recorded from 300 nm to 550 nm. Fluorescence intensity data, $(F_0 - F)/F$ were plotted against the ligand concentration to generate the binding curves, where F and F_0 represented the fluorescence intensity at 335 nm in the presence and in the absence of ligand, respectively. For EC_{50} measurements, all curves were fitted with the modified Hill equation using Igor Pro 4 as described earlier.[49–51]

Binding of resveratrol (**1**) and its derivatives (**3**, **16** and **24**) with PKCαC1B was studied by measuring the effect of the protein on the emission properties of these derivatives. Compound (**1, 3, 16** or **24**, 5 μM) was incubated in the absence or presence (20–30 μM) of PKCαC1B in buffer (50 mM Tris, 150 mM NaCl, 2 mM DTT, 50 μM $ZnSO_4$, pH 7.2) for 45 min at 25 °C and emission spectra were recorded. The solutions were excited at the corresponding absorption maximum of each compound. The wavelength maxima of the emission spectra were determined by fitting the symmetrical top of the spectra to a Gaussian function with Igor Pro 4 (WaveMetrics, Inc., Lake Oswego, OR).

4.4. Reaction of PKCαC1 with PDS (Aldrithiol-4)

Quantitative measurements of the free cysteine residues in PKCαC1 was carried out by its reaction with thiol reactive agent PDS (Aldrithiol-4).[97,98] The assay mixture (500 μl) contains 5 μM PKCαC1 and 25 μM of PDS in 5 mM phosphate buffer pH 6. After the addition of PDS, the increase in absorbance at 323 nm was measured at regular time intervals to measure the generation of 4-thiopyridone, which has a molar extinction co-efficient[98] of 19,000 M^{-1} cm^{-1} at 323 nm. Background absorption at 323 nm was recorded by using the same concentration of PDS as a reference. Stoichiometry of labeling was calculated from the amount of generated 4-thiopyridone and the amount of protein used. Labeling of the denatured protein (in 5 mM phosphate buffer, pH 6 and 8 M urea) was also determined to compare with the refolded protein.

4.5. Cell culture, transfection and compound treatment

The human embryonic kidney cell line HEK293 was maintained in DMEM supplemented with 10% foetal bovine serum, 2 mM glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C. For transfections, cells were seeded at 5–10% confluency and grown in complete medium without antibiotics for 24 h. Plasmid transfections were carried out for 48 h using lipofectamine LTX plus reagent (Invitrogen) and 6.5 μg plasmid according to manufacturer's instructions. Transfected cell starved for 12 h in serum-free DMEM and treated with resveratrol analogs (100 μM) with or without TPA (100 nM) for 2 h. Cells were washed and harvested in ice-cold phosphate-buffered saline by centrifugation at 2000 rpm for 10 min at 4 °C and subsequently used for sub-cellular fractionation.

4.6. Sub-cellular fractionation and Western blot analsysis

Cell lysis was carried out in lysis buffer (20 mM Tris–HCl, pH 7.4 plus protease inhibitor) and was briefly sonicated (4 × 5 s). Cell debris or nuclear fraction was removed by centrifugation at 3500 rpm for 10 min at 4 °C. Supernatant (100 μl) was centrifuged (200,000*g* for 1 h at 4 °C) to separate out the soluble (cytosolic) and pellet (membrane) fraction. Pellet was resuspended in 100 μl of lysis buffer with 1% Triton X-100 and sonicated three times with a 5 s pulse. Pellet fraction was incubated on ice for 1 h and centrifuged at 200,000*g* for 1 h at 4 °C. This supernatant was designated as soluble membrane fraction. Equal volume of protein was loaded for SDS–PAGE (7%) analysis. Proteins were transferred to nitrocellulose membrane and then blocked with TBST buffer (Tris-buffered saline containing 1% Tween 20) and 10 mg/ml BSA for 1 h. Membrane was washed three times with TBST buffer and incubated overnight at 4 °C with anti-EGFP antibody (1:500 dilutions). Nitrocellulose membranes were washed three times with TBST and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. An enhanced chemiluminescence detection system from Pierce (Rockford, IL) was used to visualize the labeled protein bands. The relative intensity of the protein band was calculated using alphaImager (Alpha Innotech) gel dock system.

4.7. MTT assay

The cytotoxicity of all compound was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 96-well plates. HEK293 cells were seeded (10000 cells per well) the day before treatment with different compounds (100 μM). Cells were exposed to compounds for 48 h. Cell viability was measured by MTT assay[99] using a vibrant cytotoxicity assay kit as per recommendation (Invitrogen).

4.8. Molecular docking studies

Three-dimensional structures of resveratrol and its derivatives were generated using CHEMBIODRAW ULTRA 12.0 and SYBYL 8.0. The structures were subjected to pre-dock energy minimization using 100 iterations.

NMR structure of the PKCαC1B (PDB code: 2ELI)[85] was used as the receptor template for molecular docking studies. The average

structure from the combined 20 structures for the PKCαC1B was selected using Swiss PDB Viewer 4.01.

Molecular docking was performed on SurflexDock module of SYBYL 8.0. Protomols (three dimensional space into which the substrate docked) were generated using threshold, bloat and radius values of 0.5, 2.0 and 3 Å, respectively. Residues Tyr-109, Gly-110, Ser-111, Pro-112, Thr-113, Phe-114, Leu-121, Leu-122, Tyr-123, Gly-124, Leu-125 and Gln-128 in PKCα were used for protomol generation. These residues were selected by comparing the PKC activator phorbol ester binding site in PKCδ C1B (PDB code: 1PTQ). For docking, max conformation and max rotation values were 20 and 100, respectively. Pre-dock and Post-dock energy minimization modes were also applied. Docking results were compared by the total score values. A higher total-score value represents better docking of the ligands in the receptor site.

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