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Binding of isoxazole and pyrazole derivatives of curcumin with the activator binding domain of novel protein kinase C

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ABSTRACT

The protein kinase C (PKC) family of serine/threonine kinases is an attractive drug target because of its involvement in the regulation of various cellular functions, including cell growth, differentiation, metabolism, and apoptosis. The endogenous PKC activator diacylglycerol contains two long carbon chains, which are attached to the glycerol moiety via ester linkage. Natural product curcumin (**1**), the active constituent of *Curcuma* L., contains two carbonyl and two hydroxyl groups. It modulates PKC activity and binds to the activator binding site (Majhi et al., *Bioorg. Med. Chem.* **2010**, *18*, 1591). To investigate the role of the carbonyl and hydroxyl groups of curcumin in PKC binding and to develop curcumin derivatives as effective PKC modulators, we synthesized several isoxazole and pyrazole derivatives of curcumin (**2**–**6**), characterized their absorption and fluorescence properties, and studied their interaction with the activator-binding second cysteine-rich C1B subdomain of PKCδ, PKCε and PKCθ. The EC_{50}s of the curcumin derivatives for protein fluorescence quenching varied in the range of 3–25 μM. All the derivatives showed higher binding with the PKCθC1B compared with PKCδC1B and PKCεC1B. Fluorescence emission maxima of **2**–**5** were blue shifted in the presence of the C1B domains, confirming their binding to the protein. Molecular docking revealed that hydroxyl, carbonyl and pyrazole ring of curcumin (**1**), pyrazole (**2**), and isoxazole (**4**) derivatives form hydrogen bonds with the protein residues. The present result shows that isoxazole and pyrazole derivatives bind to the activator binding site of novel PKCs and both carbonyl and hydroxy groups of curcumin play roles in the binding process, depending on the nature of curcumin derivative and the PKC isotype used.

1. Introduction

Curcumin (**1**) is a β-diketone constituent of turmeric, which is obtained from the powdered root of *Curcuma* L.[1,2] Not only it is used as a spice to give a specific flavor and yellow color to curry, which is consumed in trace quantities daily by millions of people, curcumin has also been used as a traditional medicine for liver disease (jaundice), indigestion, urinary tract diseases, rheumatoid arthritis, and insect bites.[1–3] It possesses both anti-tumor and anti-angiogenic properties. Its anti-tumor properties include growth inhibition and apoptosis induction in a variety of cancer cell lines in vitro, as well as the ability to inhibit tumorigenesis in vivo.[4–10] Curcumin also shows potent anti-inflammatory,[11,12] antioxidant,[13,14] and antimicrobial[15–18] activity. It can decrease oxidative damage, inflammation, amyloid accumulation, and is under development as a chemotherapeutic agent.[19] Curcumin is a much stronger free-radical scavenger than vitamin E,[20] protects the brain from lipid peroxidation, and scavenges nitric oxide (NO)-based radicals.[21]

Numerous analogs of curcumin have been developed for therapeutic purposes. The advantage of developing molecules around the curcumin scaffold is the lack of its toxicity. Large quantities of curcumin can be consumed without toxicity. Three phase I clinical trials have demonstrated tolerances as high as 12 g per day.[22,23] These distinctive properties make curcumin a valuable lead compound for drug development, and it remains the focus of several clinical trials.[24,25]

There are about one hundred previously reported biological targets for curcumin[24] including protein kinases,[26,27] membrane ATPases,[28–31] and transcription factors.[32,33] The positive interference of curcumin with the tumor promoting effects of phorbol esters has been attributed to its effect on the phorbol ester receptor, protein kinase C (PKC).[34] There are also well documented studies on the modulation of PKC activity by curcumin in vivo[26] and in vitro[35] using membrane-free systems.

PKC is a family of serine/threonine protein kinases involved in the regulation of various aspects of cell functions, including cell growth, differentiation, metabolism, and apoptosis.[36] PKC isoforms play an important role in the pathology of several diseases such as cancer, diabetes, stroke, heart failure, and Alzheimer's disease.[37–43] Therefore, PKC has been a subject of intensive research and drug development.[44]

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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.[45] DAG is a second messenger that is generated by the phospholipase C-catalyzed hydrolysis of membrane phosphatidylinositol-4,5-bisphosphate (PIP_2).[46] DAG selectively interacts with proteins containing a C1 domain and induces their translocation to discrete sub cellular 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 sub domains. Atypical PKCs contain a single C1 domain that does not bind DAG. Along with the PKC family, there are six additional families of proteins that contain a DAG-responsive C1 domain.[47,48] 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.[49–51]

In the present paper, we describe the design, synthesis, spectral characteristics and binding of several isoxazole and pyrazole derivatives of curcumin with the activator binding C1B subdomain of novel PKCs. Our results show that modifications of the carbonyl group to isoxazole or pyrazole change the spectral characteristics of curcumin, but still retain binding properties with the PKCs. The addition of aliphatic chain in the isoxazole or pyrazole compounds reduced the binding affinity. Molecular docking studies suggest that hydrogen bonds are formed between the hydroxyl, carbonyl or nitrogen in the heterocyclic ring of these derivatives and the protein residues.

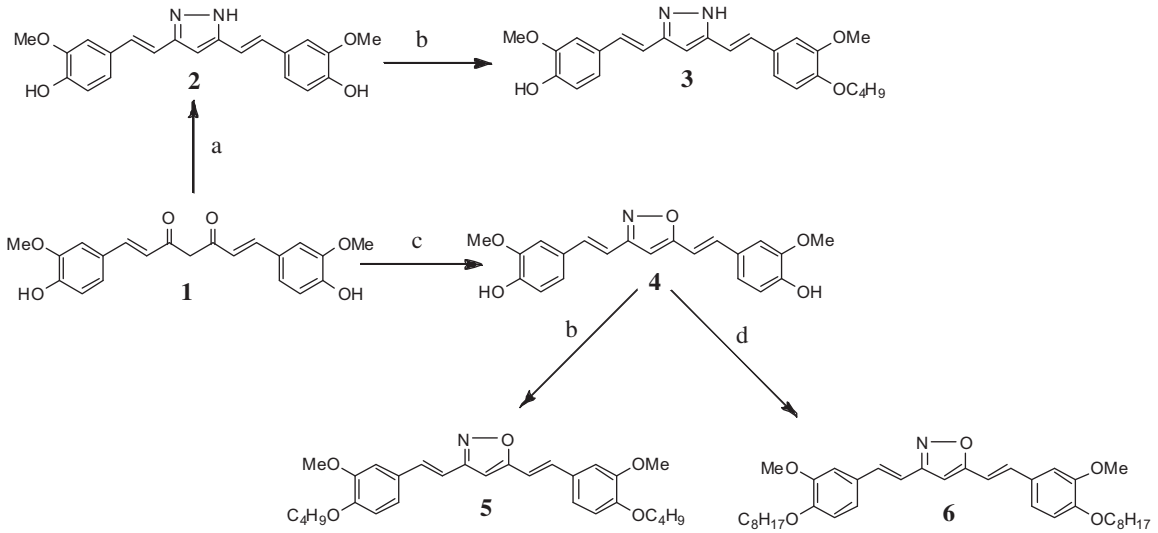
2. Chemistry

2.1. Curcumin and its cyclic derivatives

The isoxazole and pyrazole derivatives of curcumin were prepared in order to study the role of carbonyl group of curcumin in the PKC binding. Further, these derivatives were modified with aliphatic chains to study the effect of long chain in the PKC binding. Both the endogenous PKC activator diacylglycerol (DAG) and the high affinity phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) contain long aliphatic chain that facilitate the ligand for its binding to the membrane. The compounds used in this study are shown in Figure 1. Pyrazole derivative (**2**) of curcumin was prepared in good yield by heating curcumin (**1**) for 8 h with hydrazine hydrate in acetic acid, whereas isoxazole derivative (**4**) of curcumin was synthesized by treatment of curcumin with hydroxylamine hydrochloride in acetic acid at 85 °C for 6 h. Long chain derivative of curcumin-pyrazole was synthesized by refluxing curcumin-pyrazole in the presence of 1-bromobutane and K_2CO_3 in dry acetone for 24 h. Long chain derivatives of curcumin-isoxazole were prepared at refluxing condition in the presence of 1-bromooctane/1-bromobutane and K_2CO_3 in dry acetone. All the synthesized derivatives were characterized by ^1H and ^{13}C NMR and mass spectrometry.

3. Results and discussion

A large number of curcumin derivatives have been synthesized and studied for their binding and activity on receptors and proteins.[52] However, compounds designed around the scaffold of curcumin have not been explored systematically for binding and modulation of PKC activity. In our previous papers,[53,54] we showed that dietary polyphenols curcumin, resveratrol and their long chain derivatives bind to the activator-binding site in the PKCs by forming hydrogen bonds with the residues at the activator-binding site. The present investigation is a continuation of our previous study with an aim of developing subtype specific modulator of PKCs. In this study, we highlighted the role of carbonyl groups of curcumin by derivatizing them to isoxazole and pyrazole rings and studying their binding affinities with the activator-binding site of three novel PKCs, PKCδ, PKCε, and PKCθ. In order to compare the binding affinities of these derivatives with the other curcumin derivatives described earlier,[53] we used the same PKC subtypes. The isoxazole and pyrazole derivatives were further modified with the C4 aliphatic chains at the hydroxyl groups and their effects on the protein binding were also measured to determine the role of hydroxyl group in the protein–ligand interactions.



a, $NH_2NH_2.H_2O/CH_3COOH$, 8 h reflux; b, Br-C_4H_9, K_2CO_3/Acetone, reflux; c, $NH_2OH.HCl/CH_3COOH$, 85 °C, 6 h; d, Br-C_8H_{17}, K_2CO_3/Acetone, reflux.

Figure 1. Scheme outlining the synthesis of the isoxazole and pyrazole derivatives of curcumin (**1**).

Table 1
Absorption[a] and fluorescence[b] properties of curcumin and its derivatives in different solvent at 25 °C

Compound	Absorbance maximum (λ_{max}), nm				Emission maximum, nm			
	EtOH	CH_3CN	Hexane	Water	EtOH	CH_3CN	Hexane	Water
1	427	417	404	425	560	518	474 449	572
2	329	327	325	320	389	432	383 362	395
3	328	328	331	328	387	433	384 367	392
4	337	332	332	332	418	413	378 367	428
5	335	335	334	326	418	425	382 370	418
6	335	335	333	328	416	420	384 368	419

[a] concn, 2×10^{-6} M.
[b] concn, 5×10^{-6} M in water and 2×10^{-6} M in other solvents.

3.1. Absorption and emission characteristics of the curcumin derivatives

The absorption maximum of curcumin is dependent on the polarity of the solvent.[55,56] In a nonpolar solvent such as hexane, its absorption maximum is 404 nm, while in polar solvent such as ethanol, the absorption maximum is 427 nm. This strong band is assigned to be of π–π^* nature[57] based on the spectral position, spectral shift from non-polar to polar organic solvent and the intensity of the band. It was also proposed that the dipole forbidden n–π^* transition might have been buried under the strong π–π^* transition. Because curcumin can exist as an equilibrium mixture of keto and enol form, both this forms contribute to this absorption maximum. For compounds **2–6**, in which the carbonyl groups curcumin were derivatized to either cyclic isoxazole or pyrazole rings, the absorption maximum values decreased drastically in the range of 320–337 nm and showed reduced solvent sensitivity (Table 1). Figure 2A shows the absorption spectra of isoxazole derivative **4** recorded in different solvents. No significant solvent-induced wavelength shift was observed for **4**. The maximum difference of 9 nm in the absorption values was observed for **2** and **5** in water and ethanol. Although, among all the compounds examined here, the oxazole derivative showed higher absorption maximum values than the pyrazole derivative, modification of the phenolic OH group of the curcumin moiety with C4 or C8 aliphatic chain did not alter the values significantly. The presence of the two C8 aliphatic chains, however, made compound **6** poorly soluble in all the solvents tested here.

The emission maximum of curcumin is also solvent dependent, showing a single band at 572 nm in water and two maxima at 449 and 474 nm in hexane.[53,56] These emissions are believed to have characteristics of intramolecular charge transfer states.[58,59] In protic solvent such as ethanol, emission characteristics are influenced by intermolecular proton transfer, and in the aprotic solvent like

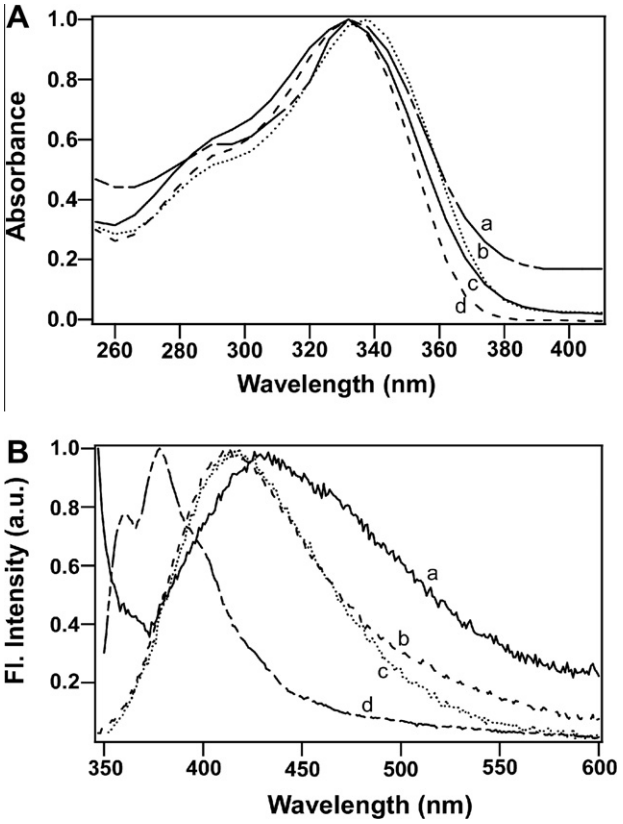


Figure 2. (A) Effect of solvent polarity on the absorption properties of curcumin and its derivatives. Normalized absorption spectra of 4,4′-((1*E*,1′*E*)-isoxazole-3,5-diylbis (ethene-2,1-diyl))bis(2-methoxyphenol) (**4**), 2×10^{-6} M in (a) water, (b) ethanol, (c) acetonitrile, and (d) hexane. (B) Effect of solvent on the emission properties of curcumin and its derivatives. Normalized fluorescence emission spectra of 4,4′-((1*E*,1′*E*)-isoxazole-3,5-diylbis(ethene-2,1-diyl))bis(2-methoxyphenol) (**4**) in (a) water, (b) acetonitrile, (c) ethanol, and (d) hexane. The concentration of **4** was 10×10^{-6} M in water and 5×10^{-6} M in other solvents.

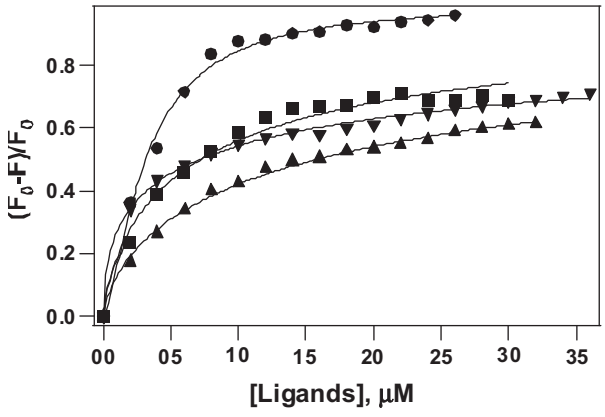


Figure 3. Binding of curcumin and its derivatives with PKCθ C1B. Plot of fluorescence intensity of PKCθ C1B (2 μM) in buffer (50 mM Tris, 150 mM NaCl, 2 mM DTT, 50 μM $ZnSO_4$, pH 7.2) in the presence of varying concentration of **2** (■), **3** (▲), **4** (●), and **5** (▼), where F and F_0 are fluorescence intensities in the presence and absence of the ligand, respectively. Solid lines indicate the fit using modified Hill equation, $f(x) = \min + (\max - \min)x^{nH}/(x^{nH} + EC_{50}^{nH})$. The corresponding EC_{50} for **2**, **3**, **4**, and **5** are 7.24, 15.86, 3.16, and 7.14 μM, respectively and the corresponding Hill coefficients (nH) are 0.74, 0.69, 1.46, and 0.51, respectively. Excitation wavelength used was 280 nm.

Table 2
EC_{50} values (in μM) and corresponding Hill coefficient (n) for the binding of curcumin and its derivatives with the C1B sub domains of PKCε, PKCδ, and PKCθ measured by fluorescence quenching. Protein concentration is 2 μM in buffer (50 mM Tris, 150 mM NaCl, 2 mM DTT, 50 μM $ZnSO_4$, pH 7.2)

Compounds	δC1B		εC1B		θC1B	
	EC_{50}	n	EC_{50}	n	EC_{50}	n
1[a]	10.67 ± 0.35	1.00	8.81 ± 0.21	1.00	11.08 ± 0.76	1.00
2	14.97 ± 0.38	0.78 ± 0.031	18.81 ± 0.39	0.70 ± 0.022	7.25 ± 0.42	0.74 ± 0.05
3	21.64 ± 0.69	0.66 ± 0.03	16.09 ± 0.18	0.75 ± 0.013	15.84 ± 0.25	0.69 ± 0.02
4	11.55 ± 0.19	1.03 ± 0.03	11.81 ± 0.20	1.14 ± 0.03	3.16 ± 0.13	1.46 ± 0.07
5	25.23 ± 0.43	0.89 ± 0.02	23.38 ± 0.52	0.76 ± 0.025	7.14 ± 0.20	0.51 ± 0.01

[a] Ref. 53.

acetonitrile and hexane, it is influenced by intramolecular proton transfer. Similar to what was observed in the absorption characteristics, the isoxazole and pyrazole compounds showed large blue shift in their emission maxima as compared to curcumin in all the solvents tested in this study. In ethanol and water, the oxazole derivatives showed higher emission maximum values as compared to the pyrazole derivatives. On the other hand, in acetonitrile, pyrazole derivatives showed higher emission maximum values as compared to the oxazole derivatives. In hexane, however, all the derivatives showed peaks at around 370 and 380 nm. For curcumin (**1**), the order of the emission maximum values was found to be water > ethanol > acetonitrile > hexane. Same order was followed for the isoxazole derivative **4**. Fig. 2B represents the emission spectra of **4** in different solvents. The isoxazole derivatives with aliphatic chains showed similar emission maxima in ethanol, acetonitrile, and water. For pyrazole derivatives, the order of emission maximum values is acetonitrile > water > ethanol > hexane. This indicates that the presence of different heteroatom can cause different solvent sensitivity in the emission properties of the curcumin derivatives.

3.2. Binding of curcumin derivatives to the PKC C1B domains

Following our previous study,[53] the binding of the isoxazole and pyrazole derivatives has been determined by fluorescence quenching methods. The C1B subdomain of the PKCδ, PKCε, and PKCθ contains a tryptophan residue, and 2, 1, and 2 tyrosine residues, respectively, which account for their intrinsic fluorescence. Compounds **2–5** quenched protein fluorescence in a concentration dependent manner and a plateau was reached at around 25–30 μM (Fig. 3). The EC_{50} values and the corresponding Hill coefficients of the quenching were determined using the modified Hill equation[49] and are shown in Table 2. In general, both isoxazole and the pyrazole derivatives showed higher binding affinity for PKCθC1B as compared with PKCδC1B and PKCεC1B. While for isoxazole (**4**), the affinity for theta was about three times higher than delta and epsilon, for pyrazole (**2**), the affinity was about two times higher. Similar higher affinity for theta was also observed earlier in the case of curcumin and its long chain derivatives.[53] Between isoxazole and pyrazole derivatives, the former showed higher affinity with all the three C1B subdomains. For example, the isoxazole (**4**) binds to theta with an EC_{50} of 3.16 μM, whereas the pyrazole (**2**) binds with an EC_{50} of 7.25 μM. It is also evident from Table 2 that the modification of isoxazole and pyrazole derivative of curcumin with aliphatic chain reduced their affinities for delta and theta. Addition of two C4 aliphatic chains in compound **5** also reduced its affinity for theta as compared with the nonchain derivative **4**, although with a lesser extent. Similarly, addition of a C4 aliphatic chain to pyrazole reduced the affinity of **3** for theta to 15.84 μM from 7.25 μM for **2**. However, different observations were made when curcumin was modified with aliphatic chains. The long chain derivatives of curcumin showed increased affinity for all the three PKC subtypes as compared with curcumin.[53]

The derivative having two C8 aliphatic chains (**6**) was not investigated for the binding studies because of its poor solubility in the concentration range tested in this study.

To further investigate the binding of the isoxazole and pyrazole derivatives of curcumin with the PKC C1B subdomains, the effect of protein on their emission maxima was studied. In the presence of 10-fold excess of PKCC1B, the emission maxima of compounds **2–5** were blue shifted. The emission maximum of **2** shifted from 395 nm in buffer to 387 nm in the presence of PKCδ C1B, and for **5**, it shifted from 415 nm in buffer to 408 nm in protein. Under similar experimental condition, curcumin showed a blue shift[53] of 10–13 nm and for a high affinity phorbol ester, sapintoxin (SAPD), the shift was even higher (15–19 nm).[53] Emission maxima of the other derivatives also were blue shifted in the presence of protein and the maximum shift of 18 nm was observed for compound **4** in PKCεC1B. (Fig. 4, Table 3). These results also indicate that isoxazole and pyrazole derivatives bind to the C1B domains of novel PKCs and the extent of binding is different for different isotype.

To gain finer details of the possible binding mode of the isoxazole and pyrazole derivatives and the interacting residues of the C1B subdomains, we performed molecular docking experiments. The molecules were docked into the activator-binding site using the phorbol ester binding residues of PKCδ and the corresponding residues of epsilon and theta at the homologous positions and the results are shown in Table 4. All the molecules showed positive total score values and most of them formed hydrogen bonds (up to ~3 Å) with the protein residues. The mode of interactions of curcumin (**1**), pyrazole (**2**) and pyrazole containing a C4 aliphatic chain (**3**) with PKCθC1B is depicted in Figure 5. Compounds **1**, **2**, and **3** interacted with the PKCθC1B by forming 3, 5, and 4 hydrogen bonds, respectively. For curcumin (**1**), all the three hydrogen bonds were formed with the 4-OH group and Gly-254. In **2**, however, three out of five hydrogen bonds, were formed with the pyrazole ring nitrogen (two with Gln-258 and one with Gly-254), and one

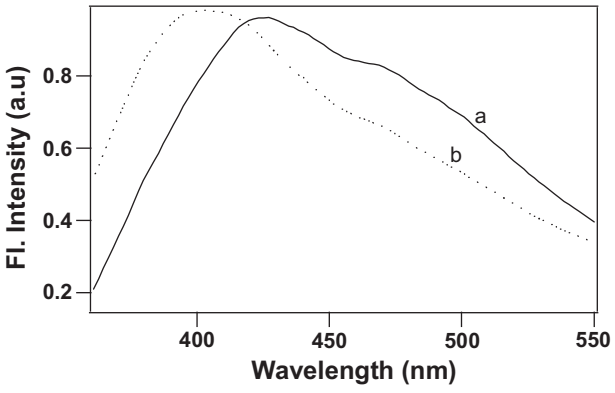


Figure 4. Blue shift in emission maxima of **4** (5×10^{-6} M) in the presence of PKCε C1B (50×10^{-6} M). Emission spectrum of **4** in (a) buffer and in (b) protein. The buffer used was 50 mM Tris, 150 mM NaCl, 2 mM DTT, 50 μM $ZnSO_4$, pH 7.2.

each with 4-OH (with Leu-252) and 4′-OH (with Lys-240). Compound **3**, with a C4 aliphatic chain, adopted a conformation in which its 4-OH formed four hydrogen bonds with Gly-254. Different types of interactions were observed in the isoxazole derivatives. Compound **4**, in which the carbonyl groups of curcumin were derivatized to an isoxazole ring, formed one hydrogen bond between the 4-OH and Gly-254. When both the hydroxyl groups were derivatized with alkyl chains resulting in **5**, no hydrogen bond formation was observed. Interestingly, the carbonyl group of curcumin formed hydrogen bonds with delta and epsilon, although no hydrogen bonds were observed with the theta. This however does not rule out the possibility of water mediated hydrogen bond between the carbonyl and PKCθC1B, which was not taken into account in the docking methods used in this study. Therefore, in general, both hydroxyl and carbonyl (or pyrazole and isoxazole rings) groups of these cyclic derivatives of curcumin are important for their interaction with PKCs and the nature of these interactions depend on the curcumin derivative and the subtype of PKC used. Using the same docking methods, it was found that one of the high-affinity activator of PKC, phorbol-13-OAc formed three hydrogen bonds with PKCθC1B (Table 4). Although we did not find a quantitative correlation between the EC_{50} values and the total score, or between the EC_{50} and the number of hydrogen bonds, which could be due to the altered conformation of both the protein and the small molecule in solution, the docking results revealed that all the derivatives interact with the protein and their possible mode of interactions.

Table 3
Emission maxima (nm)[a] of **2**–**5** in the presence of PKC C1B domains

Compound	Buffer	PKCδ	PKCε	PKCθ
1[b]	572	559	562	561
2	395	387	390	389
3	396	393	392	393
4	428	420	410	419
5	415	408	405	410

[a] **2**–**5**, 5×10^{-6} M and protein, 50×10^{-6} M. Spectra were recorded after incubating the compound for 40 min at 25 °C.
[b] Ref. 53.

Table 4
Summary of the results obtained from molecular docking. The compounds were docked into the activator binding sites of the C1B domains using Surflex dock module of Sybyl 8.0. Hydrogen bonds those are formed within the distance of 3.0 Å are reported here. Higher total score indicates higher binding probability

Compound	PKCδC1B		PKCεC1B		PKCθC1B	
	No. of H-bond	Total score	No. of H-bond	Total score	No. of H-bond	Total score
1	4	6.39	6	4.13	3	4.18
2	3	5.90	7	4.83	5	4.66
3	1	4.07	5	3.61	4	3.85
4	1	5.66	3	4.16	1	4.33
5	1	6.13	4	5.54	0	4.87
Phorbol-13-OAc	4	5.56	2	3.02	3	3.76

The isoxazole and pyrazole derivatives of curcumin were synthesized earlier to prevent tautomerization of the keto groups and to study if the electron rich isoxazole and pyrazole rings could lessen the potential for nucleophilic addition with receptor.[60,61] These analogs exhibited increased antitumor activity against hepatocellular carcinoma HA22T/VGH cell line.[60] They were also evaluated for lipoxygenase inhibitory activity,[62] cytotoxic activity,[63,64] and anti-oxidant activity,[65] anti-tumor activity against breast cancer cell lines (MCF-7, SKBR3)[66] and anti-malarial activity.[18] In the present investigation, we observed that the EC_{50} values of the isoxazole and pyrazole derivatives did show improved binding with the PKCθ as compared with curcumin, but, did not show much improvement for the PKCδ and PKCε.

The PKC C1B domains are highly conserved in their sequences and have similar overall structure. However, there is less conservation among the residues exterior of the domain that influences their interaction and affinity for the phospholipid bilayer. In fact, the lipid selectivity was found to be quite different for PKCs belonging to the same novel class.[67–69] In the present study, the binding assay was performed in the absence of any lipid, thereby ruling out the effects of lipid environment on the binding affinity of these ligands with the novel PKCs observed here. Therefore, these affinity differences are solely on the basis of the differences in the residues and surface area of the activator binding pockets.

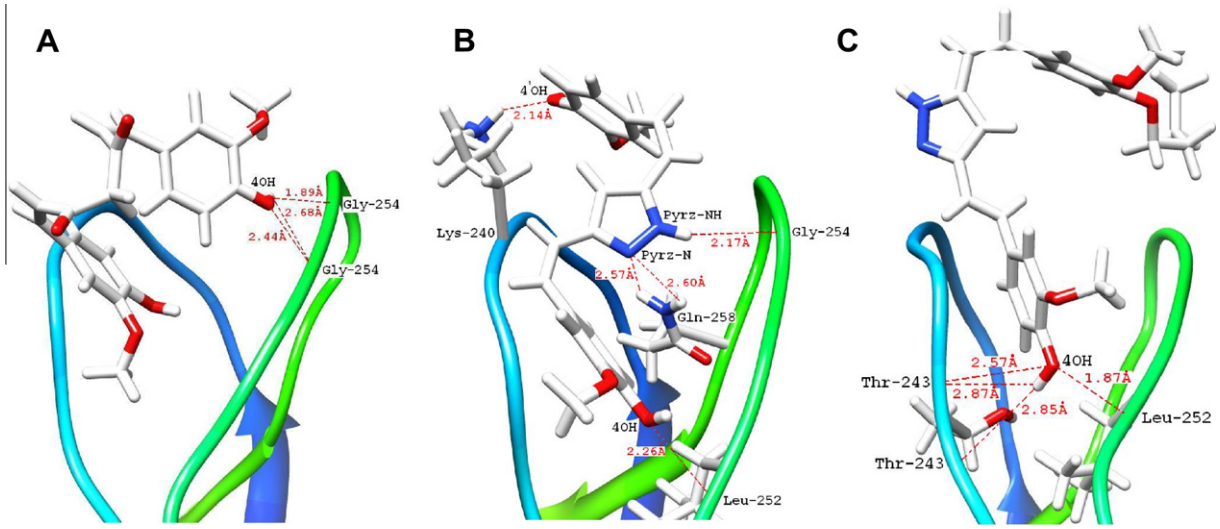


Figure 5. Docking of the isoxazole derivatives into PKCθC1B. Structure of (A) curcumin (**1**), (B) 4,4′-((1*E*,1′*E*)-(1*H*-pyrazole-3,5-diyl)bis(ethene-2,1-diyl))bis(2-methoxyphenol (**2**), and (C) 4-((*E*)-2-(5-((*E*)-4-butoxy-3-methoxystyryl)-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (**3**) docked into PKCθC1B. Only the region of the protein that interacts with the ligand is shown here. The docked structures are generated using the Surflex dock module of Sybyl 8.0 and visualized using Chimera package from the Computer Gpaphics Laboratory, University of California San Francisco, San Francisco, CA, USA. The NMR structure of PKCθC1B (PDB code: 2ENZ) has been used as the receptor template.

4. Conclusion

We described here the synthesis, spectral properties and binding of isoxazole and pyrazole derivatives of curcumin to the C1B sub domains of novel protein kinase C. Our results showed that isoxazole, pyrazole and their long chain derivatives bind to the activator-binding domain of PKC by forming hydrogen bonds with the residues at the activator binding site. Of the three novel PKCs studied here, PKCθ showed higher binding and the addition of aliphatic chain did not show any improvement in binding. Our results also indicated that both carbonyl and hydroxyl group of curcumin are important for its binding to the protein. The results presented here laid a foundation for future detailed study involving various curcumin derivatives and PKCs. On the basis of the results presented here, we are currently working towards improving the affinity and selectivity of the curcumin derivatives for the C1 domain containing proteins and also studying the effects of curcumin derivatives in membrane translocation properties of various PKC subtypes.

5. Experimental protocols

5.1. General

Curcumin was purchased from Sigma and used without further purification. Glutathione-sepharose 4B was from GE healthcare biosciences. Solvents were purchased from VWR and Fisher. All other reagents were from Sigma. 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 and ^{13}C NMR spectra were recorded on a QE-300 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. Absorption spectra were recorded on Hitachi U-2910 spectrophotometer.

5.2. Chemical synthesis

5.2.1. Synthesis of the pyrazole derivative of curcumin, (4,4′-((1*E*,1′*E*)-(1*H*-pyrazole-3, 5-diyl) bis (ethene-2,1-diyl)) bis(2-methoxyphenol) (2)

Curcumin (1.0 equiv) was dissolved in glacial acetic acid (5 mL), and hydrazine hydrate (1.1 equiv) was added to the solution. The solution was refluxed for 8 h, and then the solvent was removed under vacuum. Residue was dissolved in ethyl acetate and washed with water. Organic portion was collected, dried over sodium sulfate, and concentrated under vacuum. Crude product was purified by column chromatography on a silica column. The solvent system used was hexane/ethyl acetate, 65:35.

5.2.2. Synthesis of the pyrazole analog, 4-((*E*)-2-(5-((*E*)-4-butoxy-3-methoxystyryl)-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (3)

Mono alkylation of the pyrazole derivative (**2**) (1 equiv) was performed by addition of bromobutane (1 equiv) and K_2CO_3 (1 equiv) in acetone refluxed at 85 °C. The reaction was allowed to run for 30 h, and then washed with water. Following extraction with ethyl acetate (2 × 10 ml), the combined organic layers were evaporated under vacuum to obtain crude products. The product was purified through silica gel column chromatography using a solvent mixture (hexane:ethyl acetate:methanol = 60:27:13). Yield 57%; ^{1}H NMR (CD_3OD, 300 MHz): δ 7.13 (2H, d, J = 15.0 Hz), 7.10–7.0 (4H, m), 6.91–6.89 (2H, m), 6.75 (2H, d, J = 14.5 Hz), 6.64 (1H, s), 3.99 (2H, t), 3.89 (3H, s), 3.87 (3H, s), 1.77 (2H, m), 1.46 (2H, m), 0.89 (3H, t); ^{13}C NMR (CD_3OD, 300 MHz): δ 149.2, 147.2, 131.0, 130.4, 129.3, 122.3, 118.0, 116.6, 109.0,105.2, 68.7, 56.2, 31.6, 19.2, 14.1; MALDI: 421.3 [M+H].

5.2.3. Synthesis of the isoxazole derivative of curcumin, 4,4′-((1*E*,1′*E*)-isoxazole-3,5-diyl bis(ethene-2,1-diyl))bis(2-methoxyphenol) (4)

Hydroxylamine hydrochloride (1.1 equiv) is refluxed with 1 equiv of curcumin in the presence of acetic acid as the solvent for 6 h. The reaction mixture was evaporated to dryness; residue was dissolved in dichloromethane and washed with water. The crude product was subjected to chromatographic purification on a silica gel column. The compound was eluted with the solvent mixture (ethyl acetate/hexane/DCM, 11:7:82).

5.2.4. Synthesis of the isoxazole analog, 3,5-bis((*E*)-4-butoxy-3-methoxystyryl)isoxazole (5)

Di-alkylation of the isoxazole derivative (1 equiv) was performed by addition of bromobutane (2.2 equiv) and K_2CO_3 (2.2 equiv) in acetone. The mixture was refluxed for 24 h. After cooling the mixture to room temperature and filtering, solvent was removed under vacuum. The pure product was obtained by silica gel column chromatography (ethyl acetate:hexanes = 1:10). Yield 68%; ^{1}H NMR (CD_3OD, 300 MHz): δ 7.30 (d, J = 16.0 Hz, 2H), 7.10 (d, J = 16.0 Hz, 2H), 6.96 (d, J = 8.0 Hz, 2H), 6.91 (s, 2H), 6.83 (d, J = 8.0 Hz, 2H), 6.43 (s, 1H), 4.05 (t, 4H), 3.96 (s, 6H), 1.82 (m, 4H), 1.5 (m, 4H), 0.98 (t,6H); ^{13}C NMR (CD_3OD, 300 MHz): δ 167.7, 161.8, 149.7, 149.4, 135.2, 134.0, 128.9, 128.4, 121.0, 120.5, 113.9, 112.2, 110.9, 109.1, 108.9, 97.7, 68.1, 56.0, 19.9, 14.2; ES-MS: 478.2 [M+H].

5.2.5. Synthesis of the isoxazole analog, 3,5-bis((*E*)-3-methoxy-4-(octyloxy)styryl)isoxazole (6)

Di-alkylation of the isoxazole derivative (1 equiv) was performed by addition of bromo-octane (2.2 equiv) and K_2CO_3 (2.2 equiv) in acetone. The mixture was refluxed for 24 h. After cooling the mixture to room temperature and filtering, solvent was removed under vacuum. The pure product was obtained by silica gel column chromatography (ethyl acetate:hexanes = 1:10). Yield 72%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.40 (2H, d, J = 16.5 Hz), 7.21–7.05 (6H, m), 6.95 (2H, d, J = 16.4 Hz), 6.42 (1H, s), 4.03 (4H, t), 3.92 (3H, s), 3.91 (3H, s), 1.85 (4H, m), 1.46 (4H, m), 1.38–1.24 (16H, m), 0.86 (6H, t); ^{13}C NMR (CD_3OD, 300 MHz): δ 168.5, 162.2, 149.9, 149.6, 135.5, 134.8, 128.8, 128.5, 121.2, 120.9, 114.1, 112.6, 111.0, 109.5, 109.2, 97.7, 69.1, 56.1, 56.0, 31.9, 29.8, 29.4, 293, 29.1, 26.0, 22.7, 14.2; ES-MS: 590.9 [M+H].

5.3. Bacterial expression and purification of the PKC εC1B, δC1B, and θC1B sub domains

The PKCC1B subdomain fused with glutathione *S*-transferase (GST) was expressed in BL21 gold *Escherichia coli* and purified as described earlier.[53] Binding studies were performed with the proteins devoid of the GST tag.

5.4. Fluorescence studies

Fluorescence spectra were recorded on PTI LPS 220B equipped with temperature and stirring control systems. A 1.5-ml cuvette (Hellma) with a Teflon stopper was used for fluorescence measurements. For fluorescence quenching experiments, protein (2 μM) and varying concentration of ligands (0–35 μM) were incubated

in a buffer solution (50 mM Tris, 150 mM NaCl, 2 mM DTT, 50 μM $ZnSO_4$, pH 7.2) at 25 °C. Protein was excited at 280 nm and emission spectra were recorded from 300 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 350 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] Effect of proteins on the emission maxima of the compounds was measured for **2–5** (5 μM) in the presence of 50 μM protein in buffer (50 mM Tris, 150 mM NaCl, 2 mM DTT, 50 μM $ZnSO_4$, pH 7.2). Compounds **2–5** were incubated with the proteins for 1 h and excited at their corresponding absorption maximum. 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).

5.5. Generation of 3D models of PKC C1B sub domains and molecular docking

Three-dimensional structures of curcumin and its derivatives were generated using ChemDraw Ultra 7.0 and Sybyl 8.0. The structures were subjected to pre-dock energy minimization using 100 iterations.

Crystal structure of PKCδ C1B (PDB code: 1PTQ),[70] NMR structure of the PKCθC1B (PDB code: 2ENZ)[71] and a homology modeled structure of PKCε C1B were used as the receptors for molecular docking studies. The average structure from the combined 20 structures for the PKCθC1B was selected using Swiss PDB Viewer 4.01.

Generation of the homology model for PKCεC1B and subsequent energy minimization was done according to the methods described earlier.[53] Molecular docking was performed on SurflexDoc module of Sybyl 8.0. Protomols were generated using threshold, bloat and radius values of 0.5, 2.0, and 3 Å, respectively. Residues Tyr-239, Lys-240, Ser-241, Pro-242, Thr-243, Phe-244, Leu-251, Leu-252, Trp-253, Gly-254, Leu-255, and Glu-258 of PKC theta; Tyr-238, Met-239, Ser-240, Pro-241, Thr-242, Phe-243, Leu-250, Leu-251, Trp-252, Gly-253, Leu-254, and Gln-257 for PKC delta and Tyr-250, Lys-251, Val-252, pro-253, Thr-254, Phe-255, Leu-262, Leu-263, Trp-264, Gly-265, Leu-266, and Gln-269 for PKC epsilon were used for protomol generation. These residues were selected by comparing the PKC activator phorbol ester binding site in PKCδ C1B. For docking, max conformation and max rotation values were 20 and 100, respectively. Pre-dock and Post-dock energy minimization methods 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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