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Design and synthesis of novel 1,3,5-triphenyl pyrazolines as potential anti-inflammatory agents through allosteric inhibition of protein kinase Czeta (PKC ζ)†

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Much light has been shed on the vital role of protein kinase Czeta (PKC ζ) in NF- κ B activation and the potential use of PKC ζ inhibitors as anti-inflammatory agents. We previously reported a series of 1,3,5-trisubstituted pyrazolines as potent and selective allosteric inhibitors of PKC ζ ; in that series of compounds, the phenolic OH at the 5-phenyl was essential for binding to the PKC ζ PIF pocket. In the present study, we surprisingly found that replacing it by a halogen and at the same time moving the OH to the 3-phenyl still resulted in active compounds. An extension of this class of compounds with a new focused library is presented herein, where the phenolic OH at the 5-phenyl, which was reported to be an irreplaceable feature for activity, was moved to the 3-phenyl and replaced by halogen. The new set of compounds maintained the same level of potency against PKC ζ and selectivity against PKC isoforms, and showed reduced potency against the PIF pocket mutant PKC ζ [Val297Leu]. Of note, the repositioning of the key functional groups resulted in a marked enhancement of cellular potency. One of the most potent new PKC ζ inhibitors, **2h**, was able to suppress NO production in RAW 264.7 macrophage cells with 8 times higher efficacy than the previous series, and inhibited the NF- κ B transcriptional activity in U937 cells with a sub-micromolar IC₅₀.

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Introduction

Protein kinase Czeta (PKC ζ), together with protein kinase Ciota (PKC ι), comprises a subfamily of PKC known as atypical PKC (aPKC). They are considered atypical because they neither respond to DAG (unlike the classical and the novel PKC) nor to Ca²⁺ (unlike the classical PKC).¹ They have been reported to respond to other lipids such as phosphatidylinositols, phosphatidic acid, arachidonic acid, and ceramide.^{2,3} However, it is unclear whether some of these effects are physiologically relevant. Atypical PKCs are regulated through interaction with specific binding partners (for example Par-4 (ref. 6)) and adapters, which bind the PB1 domain on the kinase regulatory domain such as p62 and Par-6.⁴

PKC ζ is considered one of the key players in immunity and inflammation. One of the reasons behind this is the direct implication of PKC ζ in NF- κ B activation, where it was found that PKC ζ phosphorylation of the RelA subunit is required for full NF- κ B transcriptional activity *in vivo* and in cell culture experiments.⁵ This phosphorylation provides advantageous fine control of NF- κ B transcriptional activity rather than the ‘all-or-nothing’ nuclear translocation pathway. Moreover, in lung tissues, PKC ζ has an I κ B kinase (IKK) function and was found to be required for IKK activation in response to TNF α , IL-1 β , or lipopolysaccharide (LPS).⁵ Hence, pharmacological inhibition of PKC ζ could potentially block the development or progression of many diseases characterized by the expression of NF- κ B-dependent genes and gene products that contribute to the disorder. Examples of such products are cytokines and chemokines: two crucial modulators in a multitude of inflammatory and autoimmune disorders. Although further validation studies are needed, PKC ζ was proposed as a potential target for the treatment of asthma, where Th2 cells substantially contribute to airway inflammation (reviewed in ref. 6 and 7). This is in addition to the fact that PKC ζ is abundantly expressed in lung tissues. It was shown that loss of PKC ζ inhibited allergic airway disease in the ovalbumin (OVA) mouse model and reduced the

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allergic response to the OVA challenge, where mucus production was not observed in lung sections.⁸ Additionally, in OVA-challenged PKC ζ -deficient mice, IL-4, IL-5, IL-13 and eotaxin supernatant levels were highly reduced compared to similarly challenged wild type mice.⁸ Further *in vivo* studies that employed cell-permeable PKC ζ -pseudosubstrate inhibitors (PPI) pointed to a role of PKC ζ in asthmatic airway inflammation.^{9,10}

PKC ζ is also found to be largely involved in eosinophil migration in asthma, although its specific intracellular targets remain undefined.¹¹ Additionally, some studies reported PKC ζ to mediate lung inflammation in response to cigarette smoking.¹² Altogether, these data might validate PKC ζ as a promising therapeutic target in asthma and lung inflammation. However, the validity of using PPI to study PKC ζ should be taken with caution due to their possible reactivity with PKC ι or other PKCs which also have essential roles in the Th2 function. Nevertheless, the PPI results are consistent with the findings from PKC ζ -knockout mice studies. Furthermore, the evidence that PKC ζ is heavily expressed in lung extracts under resting conditions is consistent with this kinase's putative role in other pulmonary diseases like chronic obstructive pulmonary disease (COPD).¹³ We recently showed that selective PKC ζ inhibition in U937 cells, a macrophage model cell line, led to down-regulation of the expression of cytokines involved in the pathogenesis of COPD.¹⁴

In addition to the lung, a vital role of PKC ζ in the control of inflammatory disorders was also reported for the human liver.¹⁵

A limited number of compounds were reported as potent PKC ζ inhibitors; however, even the most elaborated ATP-competitive inhibitors lacked selectivity in particular towards PKC ι , the most closely related isoform to PKC ζ .¹⁶ In the context of kinase inhibitor development, allosteric inhibitors are generally believed to be more selective, because they are targeting regulatory sites that are much less conserved than the ATP-binding pocket on the catalytic domain.¹⁷ 4-Benzimidazolyl-3-phenylbutanoic acids were reported as the first class of allosteric PKC ζ inhibitors. The compounds were

shown to bind to the PIF-pocket (hydrophobic motif (HM) pocket) on the enzyme regulatory domain.¹⁸ Further development of this class led to the discovery of 1,3,5-trisubstituted and 1,3,4,5-tetrasubstituted pyrazolines as more potent allosteric PKC ζ Inhibitors. These pyrazoline derivatives showed an excellent selectivity profile towards PKC ζ over PKC ι , the most closely related atypical PKC isoform.¹⁹ In this study, we present a new extension of this class of compounds with altered positioning of the key functionalities. The synthesized compounds displayed high potency in both cell-free and cell-based assays.

Results and discussion

Compound design

Some of the previously reported 1,3,5-trisubstituted pyrazolines (exemplified by compounds A (4h in ref. 19) and B (4k in ref. 19), Fig. 1) displayed remarkable potency against recombinant PKC ζ , in particular compound A ($IC_{50} < 0.1 \mu M$). However, while these compounds efficiently suppressed the PKC ζ -mediated NF- κ B reporter gene activation in human U937 cells ($IC_{50} = 0.9 \mu M$, compound 1s in ref. 19), their potency to inhibit NO synthesis in rat RAW 264.7 cells was only moderate, thus limiting the applicability of these compounds in murine disease models. Thus, to overcome this drawback, further pharmacomodulations of this scaffold were explored.

All of the previously reported compounds had phenolic OH at the *meta* or *para* positions of the 5-phenyl as an irreplaceable feature for activity. In addition, mono or multiple halogen substitution at the 1-phenyl was shown to be essential for high potency (except at the *ortho* position). The substituents at position 3 of the pyrazoline ring elicited a range of flexibility varying from branched alkyls, substituted aryls to hetero aryls. Compound B (Fig. 1) was among the most potent compounds in the series. Compared to its close analogue compound A, compound B had an additional *para* hydroxyl group at the 3-phenyl which caused a huge boost in potency. These encouraging findings intrigued us to study whether this favourable structural feature is able to maintain the

Fig. 1 The previously reported pyrazolines, compounds A and B, as well as the newly designed compounds.

PKC ζ -inhibitory potency of the scaffold while omitting the essential hydroxyl group at the 5-phenyl. To this end, a small focused library of compounds lacking the essential hydroxyl group at the 5-phenyl was planned and synthesized (Fig. 1). In the newly designed series, the 3-phenyl is bearing the hydroxyl group under question and the 1- and 5-phenyl rings are carrying chloro substituents based on the previous biological data with compounds targeting the homologous PIF pocket in PDK1, where halogens are known to greatly enhance the potency by increasing the hydrophobic interactions.^{20,21}

Chemistry

The synthesis of the planned pyrazolines was carried out as previously reported (Scheme 1).¹⁹ Claisen-Schmidt condensation was carried out between different chlorobenzaldehyde derivatives and acetophenone analogues in a mixture of 10% aq. KOH and ethanol to yield the chalcone derivatives (C1–7). The latter were heated at 85 °C with the respective chlorophenyl hydrazine hydrochloride under an inert atmosphere using DMF as the solvent for 5 hours to give the desired pyrazolines (1a–i). A final step of methoxy deprotection was needed using BBr₃ to give the free phenolic OH (compounds 2a–h).

Biological evaluation: inhibition of recombinant PKC ζ

All of the synthesized compounds were initially screened against recombinant, full length PKC ζ at a concentration of 20 μ M. All of the synthesized phenolic compounds (2a–h) showed more than 65% inhibition (Table 1), indicating that shifting the hydroxyl function from the 5- to the 3-phenyl can indeed maintain if not surpass the potency of the previously

Table 1 Percentage inhibition of recombinant PKC ζ at 20 μ M

Cpd #	% inhibition of PKC ζ at 20 μ M ^a
1a	17.9
1b	23
1c	39
1d	30.8
1e	25.3
1f	9.6
1g	28.6
1h	39.4
1i	31
2a	69.3
2b	76.7
2c	71.8
2d	86.2
2e	85.7
2f	65.4
2g	71.1
2h	77.5

^a Values are mean values of at least two experiments; standard deviation <15%.

reported PKC ζ inhibitors. In analogy to the previously reported series, masking the phenolic OH with a methyl group significantly reduced the potency: none of the methoxy precursors (1a–h) exhibited more than 40% inhibition at the screening concentration, suggesting that in the new position, the H-bond donor properties of the phenolic OH were also important. Despite the fact that both groups can act as a HBD, the primary aromatic amino group in compound 1i failed to replace the phenolic OH in maintaining the high level of potency (compound 1i compared to compound 2f).

Compounds 2a–h were selected for the exact determination of their IC₅₀ values (Table 2). It should be mentioned

Cpd # 1/2	C	R ¹	R ²	R ³	R ⁴
a	1	4-Cl	3-OMe	4-Cl	3-OH
b	2	4-Cl	4-OMe	4-Cl	4-OH
c	3	3-Cl	4-OMe	4-Cl	4-OH
d	2	4-Cl	4-OMe	3-Cl	4-OH
e	3	3-Cl	4-OMe	3-Cl	4-OH
f	4	2-Cl	4-OMe	2-Cl	4-OH
g	5	4-Cl	2,4-Dimethoxy	4-Cl	2,4-Dihydroxy
h	6	4-Cl	3,4-Dimethoxy	4-Cl	3,4-Dihydroxy
i	7	2-Cl	4-NH ₂	2-Cl	—

Scheme 1 Reagents and conditions: (i) 10% KOH, EtOH, room temperature, overnight; (ii) 1.5 equiv. Ar-NH-NH₂-HCl, DMF, 85 °C, 5 h; (iii) 9 equiv. BBr₃, CH₂Cl₂, -78 °C then room temperature, overnight.

Table 2 Inhibition of recombinant PKC ζ , PKC ι and iNOS induction in RAW 264.7 macrophage cells

Cpd #	Cell-free assay		Inhibition of iNOS induction in RAW 264.7 macrophage cells		MTT assay using RAW 264.7
	IC ₅₀ vs. PKC ζ ^a (μ M)	% inhibition of PKC ι at 20 ^a μ M	% inhibition at 7.5 ^a μ M	IC ₅₀ ^a μ M	% living cells at 7.5 μ M of the test compound ^a
2a	6.5	9.3	45	ND	91
2b	6.2	24.94	62.4	ND	99
2c	<0.1	18.6	34	ND	96
2d	<0.1	22.8	42.8	ND	91
2e	0.4	29	31.5	ND	86
2f	21.3	30.5	34	ND	92
2g	4.3	14.85	31.4	ND	88
2h	<0.1	27.6	100	0.3	94
A (4h in ref. 18)	2.9	—	86	2.9	—
B (4k in ref. 18)	<0.1	—	87	3.1	—
Curcumin	—	—	97	0.8	—

^a Values are mean values of at least two experiments; standard deviation <15%; ND: not determined.

that the inhibition at compound concentrations below 0.1 μ M could not be measured due to the sensitivity limit of the assay. Some important SAR conclusions could be derived from the IC₅₀ values: i) the hydroxyl group at the 3-phenyl can play its essential role almost equally when it is on the *para* or *meta* positions, as underlined by compounds 2a and 2b, where both displayed almost the same IC₅₀ values (about 6 μ M). ii) Converging the hydroxyl substituents of compounds 2a and 2b on the 3,4-dihydroxy phenyl (catechol moiety) in 2h gave an IC₅₀ below 0.1 μ M, thus ranking 2h among the most potent compounds in the present series. This suggested that combining the two hydroxyl groups resulted in a marked enhancement of activity and that they are not redundant in their effect on binding to the target. In an attempt to escape the potentially metabolically unstable catechol moiety in compound 2h, the 2,4-dihydroxyphenyl analogue was synthesized, yielding compound 2g with >40-fold reduced potency. iii) In a similar fashion, the position of the halogen substituent at the 1- and 5-phenyl rings greatly influenced the potency. Decoration of one of either rings with a *meta* chloro substituent boosted the inhibitory activity to a sub 0.1 μ M level (compounds 2c and d compared to compound 2b). Having *meta* chloro substituents at both the 1- and the 5-phenyl rings led to a slight reduction of potency (compound 2e, IC₅₀ 0.4 μ M). In contrast, shifting both chloro substituents to the *ortho* position in order to bring the 1- and the 5-phenyl rings out of the plane with respect to the pyrazoline ring demonstrated a more than 50-fold drop in activity (compound 2f compared to compound 2e).

To find a potential explanation for the significant potency boosting effect of the catechol moiety, compound 2h was docked to the PIF pocket of PKC ζ which was modeled based on the PKC ι coordinates¹⁹ (PDB entry 3A8X) using MOE (Fig. 2). Similar to what has been previously found with the old series, the 3-phenyl is engaged in a cation- π interaction with Lys301 which is enhanced by the +M effect of the two hydroxyl substituents. In addition, the *m*-hydroxyl at the 3-phenyl is involved in H-bond interaction with Gln298. The

latter interaction might explain the superior potency of compound 2h over its regioisomer 2g (*o*-hydroxyl at the 3-phenyl). Altogether, the predicted tandem interaction with Lys301/Gln298, both protruding from the α C helix, potentially explains the strong impact of the catechol group on the potency. The chloro substituent that was added to the current series to replace the hydroxyl at the 5-phenyl was found to be in a suitable position and distance for halogen bonding interaction. This interaction is supported by several co-crystal structures obtained with different scaffolds targeting the PDK1 PIF pocket which possesses a similar, yet, even less accessible carbonyl at the same position. In the corresponding part of the PIF pocket, the 4-chloro always pointed towards the carbonyl oxygen.^{22,23} The docking model also explained the loss of activity seen with the 4-methoxy or 3,4-dimethoxy precursors of 2d and 2h, compounds 1d and 1h, respectively (*cf.* Table 1). It is obvious that masking of the hydroxyl function will result in a loss of the H-bond interaction with Gln298, but under identical docking conditions, the cation- π bond with Lys301 was also abandoned in favour of the now energetically preferred co-planar conformation between the pyrazoline and the dimethoxyphenyl rings (Fig. S1†) which allowed optimal conjugation with the pyrazoline double bond. This result suggested a potential cooperativity between the hydroxyl H-bond and the cation- π interaction, hence resulting in a marked loss of potency when the OH donor function is absent, like in 1d and 1h. Cooperativity between different electrostatic interactions, also extending to hydrophobic interactions, is common in protein-ligand binding and has been experimentally proven.²⁴

Selectivity vs. PKC ι and other related kinases as well as possible targeting of the PIF pocket

To test the selectivity of the synthesized compounds, compounds 2a-h were screened at 20 μ M for their ability to inhibit PKC ι , the most homologous isoenzyme, which differs by only two amino acids in the PIF pocket (Leu328 is Phe

Fig. 2 Model for the hypothetical interactions of compound **2h**. Compound **2h** (yellow) was docked into the PIF-pocket of PKC ζ which was modeled based on the PKC ι coordinates (PDB entry 3A8X) using MOE. A and B represent the binding mode from two different views to show all of the interactions. CH- π interactions are indicated by brown dashed lines; H-bonds: blue dashed line; halogen bonding interaction: cyan dashed line, with the numbers denoting the distances (in Å). C is the total view of the small lobe of the catalytic domain; colors represent the hydrophilic (magenta) and hydrophobic (green) solvent accessible surfaces.

and His289 is Asn in PKC ι , respectively). The results are shown in Table 2; none of the screened compounds showed

more than 31% inhibition at the test concentration. Hence, unlike most of the reported ATP-binding site directed inhibitors which exhibit co-inhibition of both kinases, the present compounds offer an attractive tool for selective targeting of PKC ζ . For further investigation of selectivity, compound **2h** (one of the most potent compounds in the present series) was screened against all PKC isoforms and PDK1 at 10 μ M (Table 3). Compound **2h** did not show any significant inhibition for the selected kinases, thus further reflecting the high selectivity of the synthesized compounds.

As the previously published pyrazoline series were shown to be targeting the PIF pocket, it was logical to determine the inhibitory potency of some of the active inhibitors against PKC ζ [Val297Leu] which has a mutated residue central to the PIF pocket. If the new compounds target the PKC ζ PIF pocket, a significant reduction of the potency should be observed due to steric exclusion caused by the larger leucine side chain. Indeed, according to the results shown in Table 4, the tested compounds **2b**, **2e** and **2g** showed diminished inhibition by a factor of 2–10, indicating that these compounds might also be targeting the PKC ζ -PIF pocket.

Inhibition of NO formation in RAW 264.7 macrophage cells

Numerous studies have reported that overproduction of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) has been involved in various inflammatory conditions including septic shock, tissue damage, rheumatoid arthritis, Alzheimer's disease and Parkinson's disease.^{25–27} This is in addition to its crucial role in asthma pathogenesis and pulmonary fibrosis.^{28–30} Moreover, NF- κ B has been demonstrated to be a key player in the transcriptional regulation of the murine and human iNOS gene induced by LPS and cytokines in cultured cells.^{31–34} Furthermore, PKC ζ was reported to be essential for the NF- κ B pathway activation in RAW 264.7 cells.³⁵

Table 3 Selectivity profile of **2h** vs. the PKC family and PDK1

Kinase	% inhibition at 10 μ M of 2h ^a	Kinase	% inhibition at 10 μ M of 2h ^a
PKC α	n.i.	PKC ϵ	11
PKC β I	n.i.	PKC η	n.i.
PKC β II	n.i.	PKC θ	n.i.
PKC γ	n.i.	PKN1 (PRK1)	11
PKC δ	n.i.	PDK1	1

^a Values are mean values of two experiments; standard deviation <10%; n.i.: no inhibition.

Table 4 Inhibition of PKC ζ [Val297Leu] vs. PKC ζ [wt]^a

Cpd no.	IC ₅₀ (μ M), PKC ζ [Val297Leu]	IC ₅₀ (μ M), PKC ζ [wt]	Relative potency (wt vs. mutant)
2b	20.8	6.2	3.3
2e	4.1	0.4	10.2
2g	9.7	4.3	2.2

^a Values are mean values of at least two experiments; standard deviation <12%.

Accordingly, we have reported that our previously synthesized PKC ζ inhibitors can suppress the induction of iNOS and reduce the release of its specific enzymatic product NO in the cell culture medium.¹⁹ It was also shown that the intraocular injection of a PKC ζ inhibitor peptide at 12 months of using the Goto-Kakizaki rat model for diabetes reduced iNOS expression in microglia/macrophages.³⁶ Therefore, compounds 2a–h along with curcumin were screened for their ability to inhibit NO production in RAW 264.7 cells using the Griess assay at a concentration of 7.5 μ M (Table 2). To exclude the cytotoxicity of the tested compounds against RAW 264.7 cells, an MTT assay was done in parallel with RAW 264.7 cells at the same screening concentration (Table 2). While most compounds showed a reduction in NO production between 31.5–62.5%, analogous to what we found with the previous pyrazoline series, compound 2h proved to be significantly more potent, showing full inhibition at 7.5 μ M. The IC₅₀ of 2h in suppressing NO production (0.3 μ M) indicated a 10-fold enhancement of cellular potency compared with the previously reported compound B (4k in ref. 19) which had an IC₅₀ of 3.1 μ M in the same assay. Additionally, 2h was two to three times as efficient as the reference compound curcumin: a potent inhibitor of iNOS expression and NO production.³⁷

Cellular effects on the NF- κ B signaling pathway in U937 cells

To further investigate the cellular effects of the synthesized compounds on NF- κ B transcriptional activity, the most potent compound in the inhibition of NO production (2h) was tested in U937 cells transfected with a reporter gene plasmid expressing luciferase under the control of NF- κ B response elements after induction with TNF α . The significance of this assay originates from the previous reports that U937 cells are known to have high expression of PKC ζ with an essential role in NF- κ B activation.^{38,39} Compound 2h showed 99.3% inhibition of the reporter gene activity at 10 μ M with an IC₅₀ value of 0.8 μ M, giving further evidence of the high cellular potency of the compound in the NF- κ B pathway inhibition.

Conclusions

In the present study, we pursued a strategy to modify the essential substitution pattern of our previously published 1,3,5-triphenylpyrazoline scaffold in order to find new analogues that retain the cell-free potency but show improved cellular efficacy. For this purpose, a new focused library was synthesized at which the key hydroxyl functional group was shifted from the 5- to the 3-phenyl ring. Among these new derivatives, we succeeded in identifying compound 2h as a novel lead compound, which displayed an enhanced potency to suppress the NF- κ B activity in both human U937 and rat RAW 264.7 cells, compared with the previously reported 1,3,5-trisubstituted pyrazoline analogues. In particular, the fact that compound 2h was very efficient in rat macrophages, as indicated by the inhibition of the NO production (IC₅₀ = 0.3 μ M), is a major advantage of the modified scaffold, since it opens the horizon for the evaluation of the effects of alloste-

ric PKC ζ inhibition in rat or mouse models of inflammatory diseases. This was not possible with the previous analogues, for which the best IC₅₀ observed with RAW 264.7 cells was 2.4 μ M (compound 2i in ref. 19). For *in vivo* testing, a prodrug strategy could be envisaged in the future to mask the catechol moiety by ester formation with at least one of the hydroxyl functions. Importantly, the new compounds retained the high selectivity for PKC ζ against PKC ι and other PKC isoforms and were shown to be most likely targeting the allosteric PIF-pocket using mutant PKC ζ .

Conflicts of interest

The authors declare no competing interests.

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