Diverse dynamics features of novel protein kinase C (PKC) isozymes determine selectivity of a fluorinated balanol analogue for PKCε

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Note 1: Charge state validation of 1c in novel PKC isozymes

Our previous investigation proposed that 1c has same charge state when it is bound to the ATP site of PKCε or PKA [1]. Balanoid 1c has a positive formal charge on the amine (N1) group on the azepane ring. Additionally, negative formal charges present on the phenol (C6’’OH) and carboxylic (C15’’OOH) groups of the benzophenone moiety. Thus, we assigned these charges to PKCδ-, PKCε-, PKCη-, and PKCθ-bound 1c (annotated as charge combination I). To increase the number of data points for charge state validation, we also included PKA-bound 1c to charge combination I. Each kinase-1c complex was then subjected to molecular dynamics simulation. Binding energy value of each complex was calculated using MMGBSA approach within 10-ns-sliding-window every 10 ns. The resulting ∆G\text{MMGBSA} values were evaluated further by plotting them over the MD trajectory and calculating their correlation coefficients with experiment.

The ∆G\text{MMGBSA} profile (Figure S1.A) shows that binding energy values of PKCδ-bound 1c are outliers, even though the r² plot depicts good correlations, above 0.70 from 40 to 90 ns (Figure S1.B). The experimental binding affinity order for 1c to kinase is PKCε ≈ PKCδ ≈ PKA > PKCη ≈ PKCθ. The ∆G\text{MMGBSA} values of 1c bound to PKCδ, however, are close to those of PKCη- and PKCθ-bound 1c (Figure S1.A). We then revisited the charge state of PKCδ-bound 1c. Our preceding study on charge state exploration of fluorinated balanoids suggests that local environment of the ATP site may influence the charge state of the phenol (C6’’OH) group, where it can be charged or neutral [1]. According to this result, we assigned C6’’OH neutral for PKCδ-bound 1c, whereas charge states of the remaining isozyme-bound 1c molecules are unchanged. We labeled those charge states as charge state combination II and run another MD simulation. The resulting ∆G\text{MMGBSA} profile shows the calculated binding energy values of PKCδ-bound 1c moves closer to those of PKA-bound 1c (Figure S2.A), which is expected. Moreover, after 30 ns of trajectory, correlation coefficients are above 0.80 (Figure S2.B), with
average of 0.90. Thus, in further analysis, we used 1c bearing charges on N1, C6’OH, and C15”OOH in kinases studied, except in PKCδ. In PKCδ, 1c has charges on N1 and C15”OOH.

Figure S1. (A) Trajectory of $\Delta G^{\circ}_{MMGBSA}$ of PKA- and nPKC isozymes-bound 1c using chara state combination I and (B) correlation coefficients to experimental binding energy.

Figure S2. (A) Trajectory of $\Delta G^{\circ}_{MMGBSA}$ of PKA- and nPKC isozymes-bound 1c using chara state combination II and (B) correlation coefficients to experimental binding energy.
Note 2: Analysis of per-residue binding energy decomposition at the ATP sites

General overview

Decomposition analysis of per-residue binding energy at the ATP site (Figure S7) suggests that novel PKC isozymes contribute differently to 1c. The only exception is the contribution from the adenine subsite where the benzamide moiety shows similar docking position (main paper: Figure 2). The adenine subsite residues also show similar H-bond profiles with the benzamide moiety (Table S8), particularly among novel PKC isozymes.

In the ribose subsite, two residues among kinases studied exhibit different binding energy contributions to 1c binding (Figure S7). The most dispersed contributions are ranging from -3.24 to -18.01 kcal.mol\(^{-1}\), where the strongest binding comes from Asp491 in PKCδ and the weakest from the corresponding residue Asp522 in PKC0. The second residue contributes varied binding energy values within a range -2.17 (Asp483 in PKCη) to -6.06 kcal.mol\(^{-1}\) (Asp477 in PKCδ). The binding energy strength may correlate to H-bond conservation with the amine N1 group of the azepane ring in 1c. For example, Asp491 in PKCδ has a fully conserved H-bond (99.3%), whereas Asp522 does not forms the H-bond with the amine group (Table S8).

As the regions where the flexible Gly-rich loop is mainly located, the residues of the triphosphate subsite also show diverse binding energy contributions to 1c binding among the given kinases. In particular, the invariant Lys provides the most varied contributions (-6.34 to -16.83 kcal.mol\(^{-1}\)). Additionally, this residue frequently provides the strongest binding energy contribution among the other residues in the ATP sites of the kinases, except in the ATP site of PKCδ. The other corresponding residues for Glu403 in PKCη and Glu428 in PKC0, which cause repulsive binding to 1c (Figure S7), also shows diverse binding energy values (-2.66 ± 3.60 kcal.mol\(^{-1}\)).
As mentioned in the paragraph above, the invariant Lys residues among novel PKC isozymes provides the strongest binding energy contribution to 1c. This result is similar to our previous study [2] that the invariant Lys residue in PKA and PKCε contribute a major binding contribution to the binding of balanoids studied. The invariant Lys can provide four different ways to non-covalently bind 1c, which include H-bond, charge-charge, alkyl-π hydrophobic, and cation-π interactions. The following discussions are presenting the effect of the dynamics features of nPKC isozymes on the interactions between the invariant Lys and 1c as well as binding energy contributed by this residue.

The invariant Lys residue contributions

The phenolate C6″O– group of 1c binds to the invariant Lys437 in PKCε through four ways: alkyl-π hydrophobic, cation-π, a H-bond, and charge-charge interactions. Additionally, Lys437 and carbonyl C1′=O of 1c forms a H-bond with moderate conservation (44.0%; Table S8). As a consequence, 1c acquires a very strong binding contribution of -21.39 kcal.mol\(^{-1}\) from Lys437 (Figure S7).

In PKCη and PKCθ, each of which has different dynamics behaviour to PKCε, balanoid 1c obtains weaker binding energy contributions from the invariant Lys residues. The invariant Lys in both kinases builds interactions of alkyl-π hydrophobic, cation-π, and transient H-bond with the phenolate C6″O– group in 1c. However, the H-bond between C1′=O and the invariant Lys residues (Lys363 in PKCη and Lys409 in PKCθ) is absent. Such interactions made by 1c, in the ATP site of PKCη and PKCθ, cause Lys363 and Lys409 respectively, to consecutively provide weaker binding contributions of -6.34 and -8.38 kcal.mol\(^{-1}\) (Figure S7). The greater (i.e. more negative) binding energy contribution by Lys409 is due to the H-bond between the invariant Lys and C10″-OH with conservation of 16.2% (Table S8).

The invariant Lys378 in PKCδ provides a relatively weaker binding contribution to 1c. This may be because the unique dynamics of PKCδ and the uncharged phenolic C6″OH group which
leads to fewer possibilities for interacting with Lys378. The group non-covalently binds Lys378 only through an alkyl-π hydrophobic interaction and very a transient H-bond (0.4% conservation, Table S8). As a result, 1c only obtains -8.79 kcal.mol\(^{-1}\) from Lys378 when bound to PKC\(\delta\). However, in the ribose subsite, Asp491 stabilizes the binding of 1c with -18.01 kcal.mol\(^{-1}\). This binding energy contribution is associated with a conserved H-bond (99.3%) and a salt bridge between the negatively charged side chain of Asp491 and the protonated N1 amine group in the azepane ring (main paper: Figure 5.B).

Unfavorable binding energy

Diverse dynamics of nPKC isozymes affect 1c binding in terms of shape, conformational bias, docking mode (main paper: Figure 2), and also interactions with the residues in the ATP sites. These different effects may implicate in unfavorable binding energy contributions that weaken the affinity of 1c to novel PKC isozymes. For example, Glu403 in PKC\(\eta\) contributes a repulsive binding energy of 0.14 kcal.mol\(^{-1}\) to 1c, whereas Asp421 and Glu428 in PKC\(\theta\) serves 0.01 and 0.06 kcal.mol\(^{-1}\) (Figure S7), respectively. In PKC\(\delta\), the azepane ring of 1c involves in a repulsive binding energy (0.19 kcal.mol\(^{-1}\)) with Lys475 in the ribose subsite. This unfavorable binding energy violates the strong binding energy contributed (-18.01 kcal.mol\(^{-1}\)) by Asp491 (Figure S7), reducing the binding affinity of 1c to PKC\(\delta\).

Overall decomposition analysis of per-residue binding energy suggests that although the ATP site residues among novel PKC isozymes are highly similar, the different dynamics of novel PKC isozymes result in dispersed profiles of binding energy contributions (Figure S7) to 1c. Interestingly, while the binding of 1c to PKC\(\delta\), PKC\(\eta\), and PKC\(\theta\) cause unfavourable binding interactions, PKC\(\epsilon\)-bound 1c is free from such binding interaction. Additionally, the binding of 1c to PKC\(\epsilon\) optimize the interactions with invariant Lys437 which was also reported in our previous work [2]. Hence, C5(S)-fluorine perturbation in 1c sets up properly the azepane ring and remotely drives the conformation of the benzophenone moiety to optimally interact.
with residues in the triphosphate subsite, particularly invariant Lys, which is only possible with PKCε. As a result, balanoid 1c has a high affinity and is selective to PKCε over PKA, other novel PKC isozymes, and also classical as well as atypical PKC isozymes (as suggested by $\Delta G_{MMGBSA}^r$ calculation in Table S8).
Figure S3. (A) Sequence alignment of PKA and novel PKC isozymes on their ATP sites and (B) heat map of their clustering based on Molecular Surface Electrostatic Potential (MSEP). Blue, yellow, and red-shaded colors in (A) highlight adenine subsite, ribose, and triphosphate residues, respectively. Green bars under alignment indicate the ATP site residues of PKA that are different to those of novel PKC isozymes. * - identical residue; : - conservative substitution; . – non-conservatively substituted.
Figure S4. Root mean square fluctuation (RMSF) of the apo and bound forms of nPKC isozymes. (A) and (B) are overlaid RMSF plots of nPKC isozymes in the apo and bound forms, respectively. Residues of ATP binding sites are indicated by grey-shaded bars. Residue numbering restarts from zero for all nPKC isoforms. PKCδ, ε, η, and θ are represented by green, magenta, blue, and orange color, respectively.

Figure S5. Structural mapping of principal component analysis (PCA) dynamics modelling on the apo and bound kinases. Principal modes of MD trajectories are calculated using singular value decomposition (SVD). Coloring scale of protein conformational freedom runs from red to blue which represents rigid to high flexibility.
Figure S6. Polar charts of dihedral angles on the azepane ring. Dihedral angle plots on each column represent the azepane ring of kinase-bound 1c, for the last 100 ns of MD simulation. Plots on rows monitor dihedral angles on the azepane ring that are indicated by red color atoms in the structures on the right panel.
**Figure S7.** Per-residue binding energy contributions of the ATP site of novel PKC isozymes to 1c binding.
Figure S8. Interactions of 1c with residues in the ATP sites of nPKC isoymes.
Table S1. PKC isozymes and PKA. The table contains information of Uniprot ID, template(s) used in homology modelling, and phosphorylation sites required for full activation of PKC isozymes [3].

| Isozyme | Uniprot ID | Template(s)* | Phosphorylation sites |
|---------|------------|--------------|-----------------------|
|         |            |              | Activation loop       | Turn motif | Hydrophobic motif |
| PKCα    | P17252     | 3IW4, 4RA4, 1BX6 | Thr497               | Thr638     | Ser657           |
| PKCβI   | P05771     | 2I0E, 1BX6    | Thr500               | Thr642     | Ser661           |
| PKCβII  | P05771-2   | 2I0E, 1BX6    | Thr500               | Thr642     | Ser660           |
| PKCγ    | P05129     | 2I0E, 1BX6    | Thr514               | Thr655     | Ser674           |
| PKCδ    | Q05655     | 1BX6, 5F9E    | Thr507               | Ser645     | Ser664           |
| PKCε    | Q02156     | 1BX6, 3TXO    | Thr566               | Thr710     | Ser629           |
| PKCη    | P24723     | 3TXO, 1BX6, 4RA4 | Thr513           | Thr656     | Ser675           |
| PKCθ    | Q04759     | 1BX6, 5F9E    | Thr538               | Ser676     | Ser695           |
| PKCι    | P41743     | 1BX6, 5LI9, 4DC2 | Thr412             | Thr564     | -                |
| PKCζ    | Q04759     | 1BX6, 5LI9, 4DC2 | Thr410             | Thr560     | -                |
| PKA     | P17612     | 1BX6         | Thr198               | Ser339     | -                |

* The codes listed in Template(s) column are Protein Data Bank (PDB) ID

Homology modelling and MD simulations for PKCα, βI, βII, γ, ι, and ζ were conducted as described in sub-sections of Homology modelling and Molecular dynamics simulation preparation and protocol in Materials and Methods. Phosphoryl moiety was added to each kinase as listed in Table S1.
Table S2. Cosine contents of eight principal components calculated from MD trajectories of both the *apo* and bound forms of nPKC isoforms.

| Trajectory range (ns) | Apo PKCδ | PKCε | PKCη | PKCθ | Bound PKCδ | PKCε | PKCη | PKCθ |
|-----------------------|----------|------|------|------|------------|------|------|------|
| 100-300               | 0.054    | 0.212| 0.175| 0.375| 0.368      | 0.024| 0.194| 0.385|
| 150-300               |          |      |      |      | 0.008      | 0.100|      |      |
| 175-300               |          |      |      |      | 0.030      | 0.079| 0.000| 0.000|
| 175-300               | 0.044    | 0.071| 0.035| 0.061|            | 0.030| 0.038| 0.021|
| 300-470               | 0.083    | 0.265| 0.003| 0.078|            | 0.007| 0.002| 0.000|
| 330-470               | 0.004    | 0.124| 0.048| 0.017|            | 0.001| 0.028| 0.017|
| 330-470               | 0.014    | 0.058| 0.015| 0.031|            | 0.017| 0.033| 0.021|
| 330-470               | 0.008    | 0.008| 0.049| 0.063|            | 0.013| 0.000| 0.000|

In order to obtain meaningful analyses, we monitored MD trajectories using cosine contents of eight principal components. Cosine content values range from 0 to 1, where values close to 1 is the sign of nonconverged trajectories due to random diffusion [4]. Low cosine content values may indicate converged trajectories. The majority of our MD simulations reach low cosine content after 100 ns. Based on cosine content values, we generated 300 ns trajectories for all MD simulations, except for the bound form of PKCε (470 ns).
Table S3. Sequence similarity and identity matrix of the ATP site residues of novel PKC isozymes. Matrix Global Alignment Tool (MatGAT) [5] was used to generated the matrix.

| Similarity (%) | PKCd | PKCe | PKCn | PKCθ |
|----------------|------|------|------|------|
| PKCd           |      |      |      |      |
| PKCe           |      |      | 96.4 | 96.4 |
| PKCn           | 100  | 96.4 |      | 96.4 |
| PKCθ           | 100  | 100  | 92.9 | 100  |

Table S4. Sequence similarity and identity of kinase domains of novel PKC isozymes. Matrix Global Alignment Tool (MatGAT) [5] was used to generated the matrix.

| Similarity (%) | PKCd | PKCe | PKCn | PKCθ |
|----------------|------|------|------|------|
| PKCd           |      |      |      |      |
| PKCe           | 61.9 | 61.7 | 76.3 |      |
| PKCn           | 78.5 | 76.7 | 60.4 |      |
| PKCθ           | 76.9 | 89.3 | 59.5 |      |
| PKCd           | 90.2 | 78.9 | 77.3 |      |
Table S5. Comparison between RMSF values of kinases in the apo and bound forms using a Wilcoxon sum-ranked test.

| Kinase | p-Value  |
|--------|----------|
|        |          | Kinase domain | Gly-rich loop | ATP site |
| PKCδ   | 4.21×10^{-4} | 2.16×10^{-3} | 9.73×10^{-2} |
| PKCε   | 3.49×10^{-2} | 2.16×10^{-3} | 6.27×10^{-3} |
| PKCη   | 3.32×10^{-4} | 2.16×10^{-3} | 3.12×10^{-2} |
| PKCθ   | 2.03×10^{-2} | 5.89×10^{-1} | 2.00×10^{-1} |

Table S6. RMSF changes of kinases after ligand binding to their ATP sites. Negative sign indicates RMSF reduction occurs after ligand binding, whereas a positive sign indicates an increase in RMSF after ligand binding.

| Kinase | ΔRMSF (Å)  |
|--------|------------|
|        | Kinase domain | Gly-rich loop | ATP site | triphosphate | ribose | adenine |
| PKCδ   | -0.211     | -1.067       | -0.393   | -0.839       | -0.235 | -0.058  |
| PKCε   | -0.063     | -0.847       | -0.310   | -0.743       | -0.095 | -0.028  |
| PKCη   | -0.104     | -0.205       | -0.161   | -0.403       | -0.161 | -0.038  |
| PKCθ   | 0.017      | -0.184       | 0.004    | -0.036       | 0.000  | 0.048   |
Table S7. SASA of novel PKC isozymes and PKA in the *apo* and bound forms. The values listed are average SASA values, in Å².

| State   | Isozymes | Mean  | SD    | Median | Min.   | Max.   |
|---------|----------|-------|-------|--------|--------|--------|
| *Apo*   | PKCδ     | 2614.13 | 55.73 | 2603.72 | 2479.66 | 2813.56 |
|         | PKCε     | 2598.62 | 58.72 | 2602.56 | 2405.16 | 2751.28 |
|         | PKCη     | 2682.26 | 44.97 | 2683.86 | 2531.21 | 2822.90 |
|         | PKCθ     | 2636.96 | 43.13 | 2638.59 | 2463.41 | 2769.97 |
| 1c-bound| PKCδ     | 2485.84 | 47.88 | 2484.84 | 2348.08 | 2652.92 |
|         | PKCε     | 2276.31 | 38.26 | 2273.34 | 2163.74 | 2406.57 |
|         | PKCη     | 2406.86 | 44.46 | 2405.99 | 2280.22 | 2584.70 |
|         | PKCθ     | 2476.39 | 87.25 | 2452.59 | 2301.24 | 2742.77 |
**Table S8. H-bond conservation.** The table lists H-bond persistence of 1c with the ATP site residues of nPKC isozymes.

| 1c       | PKCδ | PKCe | PKCη | PKCθ |
|----------|------|------|------|------|
|          | Residue | Residue | Residue | Residue |
| Group    | M      | R      | %    | %    | %    | %    |
| C6F1     | Az     | BB     | -    | -    | -    | Lys388 | 24.1 |
| C15"OOH | Bp     | BB     | Ser359 | 64.0 | Ser418 | 15.5 | Ser365 | 71.1 | Ser390 | 76.7 |
| C15"OOH | Bp     | SC     | 74.2 | 8.5 | 68.2 | 67.9 |
| C8"≈O   | Bp     | BB     | Phe360 | 54.2 | Phe419 | 19.9 | Phe366 | 30.8 | Phe391 | 28.3 |
| C1"≈O   | Am     | SC     | -    | Lys437 | 44.0 | -    | -    |
| C8"≈O   | Bp     | SC     | -    | -    | -    | Lys409 | 9.1 |
| C6"-OH  | Bp     | SC     | Lys378 | 0.4 | 0.5 | Lys384 | 6.8 |
| C10"-OH | Bp     | SC     | 0.2  | 1.7 | 0.7 | 16.2 |
| C10"-OH | Bp     | SC     | Glu397 | 50.0 | Glu456 | 100.0 | Glu403 | 10.3 | -    |
| C5'-OH  | Bm     | BB     | Glu428 | 64.8 | Glu487 | 89.3 | Glu434 | 87.3 | Glu459 | 88.1 |
| C5'-OH  | Bm     | BB     | Leu430 | 4.9 | Val489 | 9.9 | Val436 | 23.3 | Leu461 | 10.6 |
| N1      | Az     | BB     | Asp477 | 46.4 | Asp536 | 30.8 | Asp483 | 0.3 | Asp508 | 41.9 |
| N1      | Az     | BB     | Asn478 | 7.0  | -    | Asn484 | 0.8 | Asn509 | 1.4 |
| N1      | Az     | SC     | 7.3  | Asn537 | 15.5 | 8.1 | -    |
| C6"-OH  | Bp     | SC     | Asp491 | 6.4  | -    | -    |
| N1      | Az     | SC     | 99.3 | Asp550 | 66.0 | Asp497 | 19.2 | -    |
| C5'-OH  | Bm     | SC     | Tyr630 | 3.0  | -    | -    |
| $K_d$ (nM) | 4.9 ± 0.40 | 0.4 ± 0.01 | 13.0 ± 0 | 24.0 ± 0.50 |

*aM is moiety including Bp or benzophenone, Bm benzamide, and Az azepane, whereas Am is amide linkage.

bR is the parts of residue interacting with ligand: BB is backbone and SC is side chain.
Table S9. $\Delta G^\circ_{MGBSA}$ of natural balanol 1 and its C5(S)-fluorinated analogue (1c) to PKC isozymes and PKA.

| Kinase | $\Delta G^\circ_{MGBSA}$ (kcal.mol$^{-1}$) | (-)-Balanol 1 | 1c |
|--------|------------------------------------------|--------------|----|
| PKA    | -66.84                                   | -63.77       |    |
| PKC$\alpha$ | -67.64                                 | -36.42       |    |
| PKC$\beta$ | -62.44                                 | -48.92       |    |
| PKC$\beta$I | -56.76                                | -45.17       |    |
| PKC$\gamma$ | -73.21                                 | -56.79       |    |
| PKC$\delta$ | -72.92                                 | -45.84       |    |
| PKC$\varepsilon$ | -67.52                               | -70.35       |    |
| PKC$\eta$ | -63.34                                 | -42.35       |    |
| PKC$\theta$ | -67.51                                 | -39.77       |    |
| PKC$\iota$ | -55.47                                 | -44.28       |    |
| PKC$\zeta$ | -54.04                                 | -39.34       |    |

$\Delta G^\circ_{MGBSA}$ computations suggest that stereospecific fluorination on balanol at C5(S) results in binding selectivity to PKC$\varepsilon$. As seen in Table S6, 1c has decreased binding affinity to all PKC isozymes, except PKC$\varepsilon$. 
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