Discovery and optimization of 1,7-disubstituted-2,2-dimethyl-2,3-dihydroquinazolin-4(1H)-ones as potent and selective PKC θ inhibitorsTaisuke Katoh^{a,*}, Takafumi Takai^a, Takafumi Yukawa^a, Tetsuya Tsukamoto^a, Etsurou Watanabe^a, Hideyuki Mototani^a, Takeo Arita^a, Hiroki Hayashi^a, Hideyuki Nakagawa^a, Michael Klein^b, Hua Zou^b, Bi-Ching Sang^b, Gyorgy Snell^b, and Yoshihisa Nakada^a^aPharmaceutical Research Division, Takeda Pharmaceutical Company, Ltd., 26-1, Muraoka-higashi 2-chome, Fujisawa, Kanagawa 251-8555, Japan^bTakeda California, 10410 Science Center Drive, San Diego, CA 92121, USA^{*}Corresponding author. Tel.: +81-466-32-1171; fax: +81-466-29-4454; e-mail: taisuke.katoh@takeda.com (T.Katoh)

ARTICLE INFO

Article history:

Received

Received in revised form

Accepted

Available online

Keywords:

PKC θ inhibitors1,7-disubstituted-2,2-dimethyl-2,3-dihydroquinazolin-4(1H)-ones
inhibition of IL-2 production

ABSTRACT

A high-throughput screening campaign helped us to identify an initial lead compound (**1**) as a protein kinase C- θ (PKC θ) inhibitor. Using the docking model of compound **1** bound to PKC θ as a model, structure-based drug design was employed and two regions were identified that could be explored for further optimization, i.e., (a) a hydrophilic region around Thr442, unique to PKC family, in the inner part of the hinge region, and (b) a lipophilic region at the forefront of the ethyl moiety. Optimization of the hinge binder led us to find 1,3-dihydro-2H-imidazo[4,5-b]pyridin-2-one as a potent and selective hinge binder, which resulted in the discovery of compound **5**. Filling the lipophilic region with a suitable lipophilic substituent boosted PKC θ inhibitory activity and led to the identification of compound **10**. The co-crystal structure of compound **10** bound to PKC θ confirmed that both the hydrophilic and lipophilic regions were fully utilized. Further optimization of compound **10** led us to compound **14**, which demonstrated an improved pharmacokinetic profile and inhibition of IL-2 production in a mouse.

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1. Introduction

The protein kinase C (PKC) family is one of the serine/threonine kinases and consists of 11 isoforms grouped into classical isoforms, novel isoforms, and atypical isoforms.¹ PKC θ , classified as a novel PKC isoform, is highly expressed in T-cells and is involved in a key step in the activation of T-cells and transcription factors such as NF κ B, AP-1, and NFAT, which induce release of interleukin-2 (IL-2).² As a result, PKC θ signaling is thought to cause inflammatory diseases associated with T-cell responses. On the other hand, PKC θ -deficient mice are resistant to several models of autoimmune diseases (e.g., IBD, RA, MS, and asthma)³ and offer opportunities for T-cell modulation. Since PKC θ was identified as an attractive drug target, many papers describing PKC θ inhibitors have been published by pharmaceutical companies,⁴ although no advancement of PKC θ programs into the market has been reported. Our research program was initiated with a significant interest in the development of potent and selective PKC θ inhibitors.

A high-throughput screening campaign provided 1,7-disubstituted-2,2-dimethyl-2,3-dihydroquinazolin-4(1H)-ones, **1**, as a new class of PKC θ inhibitors. To clarify the binding mode of the inhibitors, we established a computational simulation of

compound **1** bound to PKC θ (Figure 1).⁵ The modeling revealed the main interactions between compound **1** and PKC θ , and enabled us to identify the pyrazole moiety as a bidentate hinge binder that interacted with the backbones of Glu459 and Leu461, while the carbonyl moiety interacted with the side chain of Lys409. In addition, this simulation indicated two regions that could be utilized for further optimization: a) a hydrophilic region generated by Thr442, which is unique to the PKC family, in the inner part of the hinge region, and b) a lipophilic region generated by Leu386 and Leu511 located at the forefront of the ethyl moiety. In this paper, we describe the directed synthesis of compounds designed to target these regions and the delivery of potent and selective PKC θ inhibitors.

Figure 1. Docking model of compound **1** bound to PKC θ

2. Chemistry

1,7-Disubstituted-2,2-dimethyl-2,3-dihydroquinazolin-4(1H)-one derivatives were generally synthesized as shown in **Scheme 1** or **Scheme 2**.⁶ In **Scheme 1**, 4-bromo-2-fluorobenzonitrile (**15**) was treated with the corresponding amines and hydrogen peroxide to give amide derivatives **16**. Cyclization was then performed by the reaction of **16** with 2-methoxyprop-1-ene in the presence of methanesulfonic acid. Finally, a palladium-catalyzed coupling reaction resulted in the formation of 1,7-disubstituted-2,2-dimethyl-2,3-dihydroquinazolin-4(1H)-one derivatives (**1–5**, **8**, and **10–14**). In **Scheme 2**, 4-bromo-2-fluorobenzonitrile (**15**) reacted with *tert*-butyl (3-aminopyridin-2-yl)carbamate to give the 1,3-dihydro-2H-imidazo[4,5-*b*]pyridin-2-one derivative **18**. Compound **18** was then treated with the corresponding amines and hydrogen peroxide to give amide derivatives **19**. A subsequent cyclization reaction provided 1,7-disubstituted-2,2-dimethyl-2,3-dihydroquinazolin-4(1H)-one derivatives (**6**, **7**, and **9**).

Scheme 1. General synthetic method: (i) corresponding amines R^1NH_2 , K_2CO_3 , DMSO then H_2O_2 ; (ii) 2-methoxyprop-1-ene, MsOH, acetone; (iii) boronic acid derivatives or *tert*-butyl (3-aminopyridin-2-yl)carbamate, Suzuki-Miyaura coupling or Buchwald–Hartwig coupling.

Scheme 2. General synthetic method: (i) *tert*-butyl (3-aminopyridin-2-yl)carbamate, Buchwald–Hartwig coupling; (ii) corresponding

amines R^1NH_2 , pyridine then K_2CO_3 , DMSO, H_2O_2 ; (iii) 2-methoxyprop-1-ene, MsOH, acetone.

3. Results and Discussion

Our research initially focused on the optimization of a hinge binder (**Table 1**). Initial structure-activity relationships indicated that the 3-methylpyrazole derivative **2** showed increased potency, which suggested that the methyl group was accommodated on the near side of the hinge region made up of lipophilic residues. On the other hand, the 3,5-dimethylpyrazole derivative **3** exhibited decreased potency, which suggested that one of the methyl groups was incompatible with the hydrophilic cavity around Thr442. The increased potency of **2** prompted us to design the 1H-pyrrolo[2,3-*b*]pyridine derivative **4**, resulting in enhanced PKC θ inhibition compared with compound **1**. Although **4** had a potent hinge binder, the compound exhibited significant activity against a panel of kinases.⁷ To improve kinase selectivity, our attention focused on the hydrophilic cavity restricted to some kinases including PKC θ . In our analysis of 423 kinases, only 27 kinases had inner cavities made up of hydrophilic residues.⁸ We thought that these cavities could be utilized to avoid the undesired inhibition of off-target kinases. Based on this idea, the 1,3-dihydro-2H-imidazo[4,5-*b*]pyridin-2-one derivative **5** was designed and synthesized; this hinge binder has a carbonyl group pointed toward Thr442. As expected, compound **5** displayed improved kinase selectivity with only a slight decrease in PKC θ inhibitory activity.⁹

Table 1. Optimization of hinge binders

compound	R	PKC θ IC ₅₀
1		930 nM
2		240 nM
3		1900 nM
4		60 nM
5		260 nM

After discovering a selective hinge binder, we then focused on enhancing PKC θ inhibitory activity. As mentioned above, the docking model strongly suggested that the lipophilic regions around Leu386 and Leu511 could be utilized to boost PKC θ

inhibitory activity. To fill these regions, lipophilic substituents were introduced at the 1-positions of the 2,2-dimethyl-2,3-dihydroquinazolin-4(1*H*)-one scaffolds (**Table 2**). Interestingly, a clear correlation between PKC θ inhibition potency and increased

lipophilicity of the ligands was observed among compounds **5–9**. Finally, benzyl derivative **10** showed the highest potency, although a decreased cLogP compared with cyclopentyl derivative **9** suggested that the benzyl group was involved in an additional interaction. Phenethyl derivative **11** and pyridine derivative **12** showed decreased potency compared with compound **10**. These results showed that lipophilic region occupation with a lipophilic substituent of suitable size boosted PKC θ inhibitory activity.

Table 2. Optimization of lipophilic substituents

compound	R	PKC θ IC ₅₀	Cell based assay IC ₅₀	cLogP ¹⁰
5		260 nM	>1000 nM	3.55
6		170 nM	>1000 nM	3.99
7		45 nM	>1000 nM	4.48
8		12 nM	No data	4.56
9		4.3 nM	170 nM	5.11
10		0.46 nM	16 nM	4.72
11		300 nM	>1000 nM	5.12
12		150 nM	No data	3.23

The co-crystal structure of compound **10** with PKC θ was successfully obtained; the binding mode was identical to that predicted by our docking model.¹¹ The structure revealed hydrogen-bonding interactions between **10** and the hinge Glu459, Leu461, and catalytic Lys409 residues, as well as effective occupation of both the hydrophilic and lipophilic regions. The carbonyl group of the hinge binder was pointed towards the inner part of the hinge region around Thr442 and occupied the hydrophilic region. In the region around the catalytic Lys409, there was an interaction between the side chain of Lys409 and the carbonyl group that was extended from the dihydroquinazolinone scaffold. The benzyl group of compound **10** was accommodated in the lipophilic region, which was composed of the lipophilic residues Leu386, Phe391, Val394, Leu511, and Phe664. The distance between the carbon atom on 7-position of the hinge binder and the center of the benzene ring was 4.0 Å, which might indicate an intramolecular CH- π interaction.¹² This interaction led to a conformational lock, which had a positive effect on PKC θ inhibitory ability through a decrease in the entropic penalty required to achieve the active conformation.¹³ This co-crystal structure could help explain the results of our initial structure-activity relationship.

Figure 2. Co-crystallized structure of compound **10** bound to PKC θ .

Although compound **10** was a highly potent PKC θ inhibitor, it showed low oral bioavailability in a mouse (**Table 3**). The poor pharmacokinetic properties in the mouse prevented us from carrying out further in vivo evaluation. We assume that metabolic instability could be the cause of the poor profiles observed, because the benzylic position has previously been identified as being metabolically unstable.¹⁴ The structure of the complex formed between compound **10** and PKC θ suggested that the introduction of a small substituent into the benzylic position could be accommodated and would protect the site from metabolism without impairing the PKC θ potency. Based on this hypothesis, a methyl group was introduced at the benzylic position of compound **10**. Profiling demonstrated that the (*R*)-methyl derivative **14** had the best potency, and the metabolic stability and pharmacokinetic profile were both improved over the parent compound.

Compound **14** was selected for further characterization and showed good potency in a cell-based assay and good selectivity over a wide range of kinases, including the PKC subfamily (30 kinases) (**Table 4**).

Compound **14** was evaluated in a human whole blood IL-2 assay and in an in vivo PD model in a mouse to prove its

physiological relevance. Compound **14** showed good potency in a dose-dependent manner in the human whole blood IL-2 assay. In a mouse model of anti-CD3 antibody-induced IL-2 release, single doses of compound **14** administered orally at 10 mg/kg and 100 mg/kg demonstrated dose-dependent inhibition of IL-2 production (**Figure 3**). The concentration of compound **14** at 4

h after oral administration was 826 ng/mL on average. This meant the compound exposure was about 2000 nM, which covered IC₅₀ value of a mouse WB IL-2 assay (IC₅₀ = 682 nM).

Table 3. Effect of introducing methyl group at benzylic position of compound **10**

compounds	R	PKC θ IC ₅₀	Metabolic stability in mouse liver microsomes (μ L/min/mg)	Mouse PK ¹⁵		
				CL (ml/h/kg)	AUC (po) (ng·h/ml)	BA (%)
10		0.46 nM	160	8570	16	12
13		100 nM	140	6395	11	7
14		0.25 nM	30	1641	172	28

Table 4. Cell-based assay and general kinase selectivity profile for compound **14**

Cell based assay (IC ₅₀)	Kinase selectivity profile (30 kinases)		
11 nM	IC ₅₀ > 1000 nM (21 kinases)	IC ₅₀ > 100 nM (3 kinases)	IC ₅₀ > 10 nM (6 kinases)
	EGFR, VEGFR2, IRK, EphA5, JAK1, IRAK4, MEK1, ASK1, CK1 δ , Aurora-B, ROCK1, CHK1, MAPKAPK2, ERK1, CDK2, p38 α , CDC7, PLK1, PI3K α , ROCK2, PKC ζ ,	c-Met, SRC, FAK	PKC α , PKC β , PKC δ , CLK, GSK-3 β , TrkA

Figure 3. Human whole blood IL-2 and Mouse IL-2 PD assays of compound **14**

4. Conclusions

In summary, a new class of PKC θ inhibitors has been described in this paper. Based on the docking model of our initial lead compound, **1**, bound to the PKC θ protein, a design strategy to increase kinase selectivity and PKC θ inhibitory activity was developed. To increase the selectivity over general kinases, the hydrophilic region around Thr442, unique to the PKC family, was targeted, resulting in the discovery of a unique hinge binder, 1,3-dihydro-2*H*-imidazo[4,5-*b*]pyridin-2-one. To increase the potency of the PKC θ inhibitors, their lipophilic regions were targeted, which resulted in an increase in PKC θ potency up to IC₅₀ = 0.46 nM. Further optimization to improve the pharmacokinetic profile provided the (*R*)-methyl derivative **14**. Compound **14** was evaluated in more physiologically relevant assays, viz., human whole-blood IL-2 and mouse IL-2 PD assays, and demonstrated dose-dependent inhibition of IL-2 production. We confirmed that the PKC θ inhibitor contributed to the inhibition of IL-2 production in the T-cell signaling pathway and demonstrated the potential for the effective treatment of inflammatory diseases associated with T-cell responses.

5. Experimental

5.1. Compound synthesis

5.1.1. General experimental

All commercially available solvents and reagents were used without further purification. ¹H NMR spectra were recorded on a Bruker DPX300 (300 MHz for ¹H NMR) instrument. Chemical shifts are reported as δ values (ppm) downfield from internal TMS. Peak multiplicities are expressed as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; td, triplet of doublets; ddd, doublet of doublet of doublets; quin, quintet; br s, broad singlet; m, multiplet. Coupling constants (*J* values) are given in hertz (Hz). Chromatographic purification was carried out on Universal columns (SI or NH, Yamazen). LC-MS spectra were obtained on a Shimadzu Corporation LC-MS system (LCMS-2020). The method employed to determine purity utilized a Shimadzu UFLC instrument with an L-column 2 ODS 2 μ m, 2.1 mm $\times$ 30 mm. All analyzed compounds were >95% pure unless otherwise indicated. Reaction progress was determined using thin layer chromatography (TLC) analysis on silica gel 60 F₂₅₄ plates (Merck) or NH TLC plates (Fuji Silysia Chemical Ltd.). Microwave reactions were performed in a commercially available Biotage® initiator. The following abbreviations are used: DMSO, dimethyl sulfoxide; DMSO-*d*₆, dimethyl sulfoxide-*d*₆; EtOAc, ethyl acetate; MeOH, methanol; ^tBuOH, *tert*-butyl alcohol; Pd₂(dba)₃, tris(dibenzylideneacetone)dipalladium(0); Ruphos, 2-dicyclohexylphosphino-2',6'-diisopropoxy-1,1'-biphenyl; ^tBuONa, sodium *tert*-butoxide; H₂O₂, hydrogen peroxide; K₂CO₃, potassium carbonate; NaHCO₃, sodium hydrogen carbonate; Na₂SO₄, sodium sulfate; Na₂S₂O₃, sodium thiosulfate.

5.1.2. General procedure in Scheme 1

Compound **14** was synthesized as described below.

5.1.2.1. 4-Bromo-2-[(1*R*)-1-phenylethyl]amino}benzamide (**16**)

To a mixture of 4-bromo-2-fluorobenzonitrile (**15**) (1.50 g, 7.50 mmol) in DMSO (10 mL) was added (1*R*)-1-phenylethanamine (1.24 mL, 9.75 mmol) and K₂CO₃ (2.07 g,

15.0 mmol). The mixture was stirred at 100 °C overnight. After being cooled to room temperature, 35% aqueous H₂O₂ solution (3.64 mL, 37.5 mmol) was added to the reaction mixture. The mixture was stirred at room temperature for 2 h and poured into water. The precipitate was collected by filtration and dried in vacuo to afford 4-bromo-2-[(1*R*)-1-phenylethyl]amino}benzamide (2.22 g, 93%) as a yellow solid. ¹H NMR (DMSO-*d*₆) δ : 1.27–1.45 (3H, m), 4.44–4.86 (1H, m), 6.18–6.60 (1H, m), 6.61–6.74 (1H, m), 7.07–7.36 (6H, m), 7.41–7.61 (1H, m), 7.69–8.18 (1H, m), 8.70–9.01 (1H, m). LC-MS(ESI) *m/z* = 317.0, 319.0 [M-H][−].

5.1.2.2. 7-Bromo-2,2-dimethyl-1-[(1*R*)-1-phenylethyl]-2,3-dihydroquinazolin-4(1*H*)-one (**17**)

To a solution of 4-bromo-2-[(1*R*)-1-phenylethyl]amino}benzamide (**16**) (1.0 g, 3.13 mmol) in acetone (20 mL) was added 2-methoxyprop-1-ene (1.48 mL, 15.7 mmol) and methanesulfonic acid (0.020 mL, 0.31 mmol). The mixture was stirred at room temperature for 1 h and then concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with EtOAc in hexane) to give 7-bromo-2,2-dimethyl-1-[(1*R*)-1-phenylethyl]-2,3-dihydroquinazolin-4(1*H*)-one (0.80 g, 71%) as a pale yellow solid. ¹H NMR (DMSO-*d*₆) δ : 1.51 (3H, s), 1.63 (3H, s), 1.72 (3H, d, *J* = 6.8 Hz), 5.21 (1H, q, *J* = 6.8 Hz), 6.40 (1H, d, *J* = 1.5 Hz), 6.80 (1H, dd, *J* = 8.1, 1.7 Hz), 7.18–7.30 (2H, m), 7.36–7.40 (3H, m), 7.61 (1H, d, *J* = 8.3 Hz), 8.30 (1H, s). LC-MS (ESI) *m/z* = 359.2, 361.2 [M+H]⁺.

5.1.2.3. 2,2-Dimethyl-7-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridin-1-yl)-1-[(1*R*)-1-phenylethyl]-2,3-dihydroquinazolin-4(1*H*)-one (**14**)

To a solution of *tert*-butyl (3-aminopyridin-2-yl)carbamate (105 mg, 0.50 mmol) in ^tBuOH (3 mL) was added 7-bromo-2,2-dimethyl-1-[(1*R*)-1-phenylethyl]-2,3-dihydroquinazolin-4(1*H*)-one (**17**) (150 mg, 0.42 mmol), Pd₂(dba)₃ (38.2 mg, 0.04 mmol), ^tBuONa (80 mg, 0.84 mmol), and RuPhos (78 mg, 0.17 mmol). The mixture was stirred at 100 °C for 3 h under an Ar atmosphere. The mixture was filtered and the filtrate was concentrated in vacuo. The residue was purified by column chromatography (NH silica gel, eluted with MeOH in EtOAc). The solid obtained was washed with EtOAc to give 2,2-dimethyl-7-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridin-1-yl)-1-[(1*R*)-1-phenylethyl]-2,3-dihydroquinazolin-4(1*H*)-one (103 mg, 60%) as a pale yellow solid. ¹H NMR (DMSO-*d*₆) δ : 1.57 (3H, s), 1.66–1.81 (6H, m), 5.23 (1H, q, *J* = 7.1 Hz), 5.95 (1H, d, *J* = 6.8 Hz), 6.32 (1H, d, *J* = 1.9 Hz), 6.72 (1H, dd, *J* = 7.9, 5.3 Hz), 6.86 (1H, dd, *J* = 8.3, 1.9 Hz), 7.30–7.49 (5H, m), 7.79–7.94 (2H, m), 8.30 (1H, s), 11.79 (1H, s). LC-MS (ESI) *m/z* = 414.3 [M+H]⁺.

The compounds **1–5**, **8**, and **10–13** were prepared in a manner similar to that described for compound **14**.

5.1.2.4. 1-Ethyl-2,2-dimethyl-7-(1*H*-pyrazol-4-yl)-2,3-dihydroquinazolin-4(1*H*)-one (**1**)

¹H NMR (DMSO-*d*₆) δ : 1.17 (3H, t, *J* = 6.8 Hz), 1.43 (6H, s), 3.41 (2H, d, *J* = 6.8 Hz), 6.89 (1H, s), 6.96 (1H, d, *J* = 7.9 Hz), 7.66 (1H, d, *J* = 7.9 Hz), 7.82–8.09 (2H, m), 8.25 (1H, br s), 13.00 (1H, br s). LC-MS (ESI) *m/z* = 271.3 [M+H]⁺.

5.1.2.5. 1-Ethyl-2,2-dimethyl-7-(3-methyl-1H-pyrazol-4-yl)-2,3-dihydroquinazolin-4(1H)-one (**2**)

Compound **2** was obtained (13%) as a white solid from a coupling reaction using *tert*-butyl 3-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole-1-carboxylate, 7-bromo-1-ethyl-2,2-dimethyl-2,3-dihydroquinazolin-4(1H)-one, bis[di-*tert*-butyl(4-dimethylaminophenyl)phosphine]dichloropalladium(II), and K₂CO₃ in 1,2-dimethoxyethane and water, followed by a Boc deprotection reaction using 4M HCl-EtOAc solution.

¹H NMR (DMSO-*d*₆) δ: 1.18 (3H, t, *J* = 6.8 Hz), 1.44 (6H, s), 2.39 (3H, s), 3.36–3.43 (2H, m), 6.72 (1H, s), 6.81 (1H, dd, *J* = 7.9, 1.1 Hz), 7.68 (1H, d, *J* = 7.9 Hz), 7.84 (1H, br s), 7.97 (1H, s), 12.76 (1H, s). LC-MS (ESI) *m/z* = 285.3 [M+H]⁺.

5.1.2.6. 7-(3,5-Dimethyl-1H-pyrazol-4-yl)-1-ethyl-2,2-dimethyl-2,3-dihydroquinazolin-4(1H)-one (**3**)

Compound **3** was obtained (62%) as a white solid from a coupling reaction using *tert*-butyl 3,5-dimethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole-1-carboxylate, 7-bromo-1-ethyl-2,2-dimethyl-2,3-dihydroquinazolin-4(1H)-one, bis[di-*tert*-butyl(4-dimethylaminophenyl)phosphine]dichloropalladium(II), and K₂CO₃ in 1,2-dimethoxyethane and water, followed by a Boc deprotection reaction using 4M HCl-EtOAc solution.

¹H NMR (DMSO-*d*₆) δ: 1.10–1.22 (3H, m), 1.44 (6H, s), 2.23 (6H, br s), 3.26–3.41 (2H, m), 6.54 (1H, d, *J* = 1.1 Hz), 6.64 (1H, dd, *J* = 7.9, 1.1 Hz), 7.70 (1H, d, *J* = 7.9 Hz), 7.98 (1H, s), 12.36 (1H, br s). LC-MS (ESI) *m/z* = 299.3[M+H]⁺.

5.1.2.7. 1-Ethyl-2,2-dimethyl-7-(1H-pyrrolo[2,3-*b*]pyridin-3-yl)-2,3-dihydroquinazolin-4(1H)-one (**4**)

Compound **4** was obtained (11%) as a pale yellow solid from a coupling reaction using *tert*-butyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrrolo[2,3-*b*]pyridine-1-carboxylate, 7-bromo-1-ethyl-2,2-dimethyl-2,3-dihydroquinazolin-4(1H)-one, bis[di-*tert*-butyl(4-dimethylaminophenyl)phosphine]dichloropalladium(II), and K₂CO₃ in 1,2-dimethoxyethane and water, followed by a Boc deprotection reaction using 4M HCl-EtOAc solution.

¹H NMR (DMSO-*d*₆) δ: 1.22 (3H, t, *J* = 7.2 Hz), 1.47 (6H, s), 3.45 (2H, q, *J* = 6.9 Hz), 6.97 (1H, s), 7.08 (1H, dd, *J* = 8.1, 1.3 Hz), 7.18 (1H, dd, *J* = 7.9, 4.5 Hz), 7.75 (1H, d, *J* = 7.9 Hz), 7.92–8.02 (2H, m), 8.19–8.34 (2H, m), 12.02 (1H, br s). LC-MS (ESI) *m/z* = 321.3[M+H]⁺.

5.1.2.8. 1-Ethyl-2,2-dimethyl-7-(2-oxo-2,3-dihydro-1H-imidazo[4,5-*b*]pyridin-1-yl)-2,3-dihydroquinazolin-4(1H)-one (**5**)

Compound **5** was obtained (30%) as a white solid. ¹H NMR (DMSO-*d*₆) δ: 1.15 (3H, t, *J* = 6.6 Hz), 1.47 (6H, s), 3.33–3.41 (2H, m), 6.80–6.94 (2H, m), 7.04 (1H, dd, *J* = 7.7, 5.1 Hz), 7.41 (1H, dd, *J* = 7.9, 1.1 Hz), 7.84 (1H, d, *J* = 8.3 Hz), 7.99 (1H, dd, *J* = 5.3, 1.1 Hz), 8.16 (1H, s), 11.86 (1H, br s). LC-MS (ESI) *m/z* = 338.3[M+H]⁺.

5.1.2.9. 2,2-Dimethyl-7-(2-oxo-2,3-dihydro-1H-imidazo[4,5-*b*]pyridin-1-yl)-1-phenyl-2,3-dihydroquinazolin-4(1H)-one (**8**).

Compound **8** was obtained (32%) as a pale yellow solid. ¹H NMR (DMSO-*d*₆) δ: 1.43 (6H, s), 6.13 (1H, d, *J* = 1.9 Hz), 6.90–7.01 (2H, m), 7.21 (1H, dd, *J* = 7.7, 1.3 Hz), 7.34–7.46 (3H, m), 7.48–7.59 (2H, m), 7.86–7.97 (2H, m), 8.43 (1H, s), 11.78 (1H, s). LC-MS (ESI) *m/z* = 386.2 [M+H]⁺.

5.1.2.10. 1-Benzyl-2,2-dimethyl-7-(2-oxo-2,3-dihydro-1H-imidazo[4,5-*b*]pyridin-1-yl)-2,3-dihydroquinazolin-4(1H)-one (**10**).

Compound **10** was obtained (42%) as a pale yellow solid. ¹H NMR (DMSO-*d*₆) δ: 1.55 (6H, s), 4.56 (2H, s), 6.19 (1H, dd, *J* = 7.9, 1.3 Hz), 6.52 (1H, d, *J* = 1.5 Hz), 6.68 (1H, dd, *J* = 7.9, 5.3 Hz), 6.95 (1H, dd, *J* = 8.3, 1.5 Hz), 7.31–7.44 (5H, m), 7.85 (1H, d, *J* = 8.3 Hz), 7.90 (1H, dd, *J* = 5.3, 1.3 Hz), 8.34 (1H, s), 11.82 (1H, br s). LC-MS (ESI) *m/z* = 400.3 [M+H]⁺.

5.1.2.11. 2,2-Dimethyl-7-(2-oxo-2,3-dihydro-1H-imidazo[4,5-*b*]pyridin-1-yl)-1-(2-phenylethyl)-2,3-dihydroquinazolin-4(1H)-one (**11**).

Compound **11** was obtained (64%) as a pale yellow solid. ¹H NMR (DMSO-*d*₆) δ: 1.44 (6H, s), 2.89 (2H, t, *J* = 7.7 Hz), 3.45 (2H, t, *J* = 7.6 Hz), 6.94 (1H, dd, *J* = 8.3, 1.5 Hz), 7.02–7.10 (2H, m), 7.17–7.33 (5H, m), 7.53 (1H, dd, *J* = 7.7, 1.3 Hz), 7.87 (1H, d, *J* = 8.3 Hz), 8.02 (1H, dd, *J* = 5.3, 1.1 Hz), 8.17 (1H, s), 11.94 (1H, s). LC-MS (ESI) *m/z* = 414.3 [M+H]⁺.

5.1.2.12. 2,2-Dimethyl-7-(2-oxo-2,3-dihydro-1H-imidazo[4,5-*b*]pyridin-1-yl)-1-(pyridin-3-ylmethyl)-2,3-dihydroquinazolin-4(1H)-one (**12**).

Compound **12** was obtained (3%) as a white solid. ¹H NMR (DMSO-*d*₆) δ: 1.55 (6H, s), 4.62 (2H, s), 6.40 (1H, dd, *J* = 7.8, 1.2 Hz), 6.60 (1H, d, *J* = 1.5 Hz), 6.77 (1H, dd, *J* = 7.8, 5.2 Hz), 6.96 (1H, dd, *J* = 8.3, 1.7 Hz), 7.41 (1H, dd, *J* = 7.7, 4.7 Hz), 7.74 (1H, d, *J* = 7.9 Hz), 7.81–8.02 (2H, m), 8.37 (1H, s), 8.49–8.68 (2H, m), 11.83 (1H, s). LC-MS (ESI) *m/z* = 401.3 [M+H]⁺.

5.1.2.13. 2,2-Dimethyl-7-(2-oxo-2,3-dihydro-1H-imidazo[4,5-*b*]pyridin-1-yl)-1-[(1*S*)-1-phenylethyl]-2,3-dihydroquinazolin-4(1H)-one (**13**).

Compound **13** was obtained (70%) as a pale yellow solid. ¹H NMR (DMSO-*d*₆) δ: 1.57 (3H, s), 1.66–1.81 (6H, m), 5.23 (1H, d, *J* = 7.1 Hz), 5.95 (1H, d, *J* = 6.8 Hz), 6.32 (1H, d, *J* = 1.9 Hz), 6.72 (1H, dd, *J* = 7.9, 5.3 Hz), 6.86 (1H, dd, *J* = 8.3, 1.9 Hz), 7.30–7.49 (5H, m), 7.79–7.94 (2H, m), 8.30 (1H, s), 11.79 (1H, s). LC-MS (ESI) *m/z* = 414.3 [M+H]⁺.

5.1.3. General procedure in Scheme 2

Compound **9** was synthesized as described below.

5.1.3.1. 2-Fluoro-4-(2-oxo-2,3-dihydro-1H-imidazo[4,5-*b*]pyridin-1-yl)benzonitrile (**18**)

To a solution of *tert*-butyl (3-aminopyridin-2-yl)carbamate (500 mg, 2.39 mmol) in ¹BuOH (5 mL) was added Pd₂(dba)₃ (182 mg, 0.20 mmol), 4-bromo-2-fluorobenzonitrile (**15**) (398 mg, 1.99 mmol), ¹BuONa (383 mg, 3.98 mmol) and RuPhos (372 mg, 0.80 mmol). The mixture was stirred at 100 °C for 3 h under an Ar atmosphere. The mixture was filtered and the filtrate was concentrated in vacuo. The residue was purified by column chromatography (NH silica gel, eluted with MeOH in EtOAc). The resultant solid was recrystallized from EtOH-EtOAc-hexane to give 2-fluoro-4-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridin-1-yl)benzonitrile (373 mg, 74%) as a pale yellow solid. ¹H NMR (DMSO-*d*₆) δ: 7.07 (1H, dd, *J* = 7.9, 5.3 Hz), 7.59 (1H, d, *J* = 7.9 Hz), 7.68–7.74 (1H, m), 7.87 (1H, dd, *J* = 11.0, 1.9 Hz), 8.04 (1H, d, *J* = 4.9 Hz), 8.08–8.15 (1H, m), 12.08 (1H, br s). LC-MS (ESI) *m/z* = 253.0 [M-H][−].

5.1.3.2. 2-[(Cyclopentylmethyl)amino]-4-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridin-1-yl)benzamide (**19**)

A mixture of 2-fluoro-4-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridin-1-yl)benzonitrile (**18**) (100 mg, 0.39 mmol), 1-cyclopentylmethanamine (117 mg, 1.18 mmol), and pyridine (4 mL) was heated at 140 °C for 2 h under microwave irradiation. The mixture was then concentrated in vacuo. To the residue was added DMSO (4 mL), K₂CO₃ (109 mg, 0.79 mmol), and 35% aqueous H₂O₂ solution (191 mg, 1.97 mmol). The mixture was stirred at 50 °C for 5 h, then quenched with a saturated aqueous Na₂S₂O₃ solution and extracted with EtOAc and 2-propanol. The organic layer was separated, washed with brine, dried over Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with MeOH in EtOAc) to give 2-[(cyclopentylmethyl)amino]-4-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridin-1-yl)benzamide (61 mg, 44%) as a white solid. ¹H NMR (DMSO-*d*₆) δ: 1.20–1.32 (2H, m), 1.43–1.68 (4H, m), 1.70–1.84 (2H, m), 2.07–2.23 (1H, m), 3.00–3.05 (2H, m), 6.66 (1H, dd, *J* = 8.5, 2.1 Hz), 6.79 (1H, d, *J* = 1.9 Hz), 7.03 (1H, dd, *J* = 7.7, 5.1 Hz), 7.23 (1H, br s), 7.38 (1H, dd, *J* = 7.9, 1.1 Hz), 7.76 (1H, d, *J* = 8.3 Hz), 7.91 (1H, br s), 7.98 (1H, dd, *J* = 5.3, 1.1 Hz), 8.46 (1H, t, *J* = 5.1 Hz), 11.84 (1H, br s). LC-MS (ESI) *m/z* = 352.3 [M+H]⁺.

5.1.3.3. 1-(Cyclopentylmethyl)-2,2-dimethyl-7-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridin-1-yl)-2,3-dihydroquinazolin-4(1*H*)-one (**9**)

A mixture of 2-[(cyclopentylmethyl)amino]-4-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridin-1-yl)benzamide (**19**) (60 mg, 0.17 mmol), *p*-toluenesulfonic acid monohydrate (3 mg, 0.02 mmol), acetone (2 mL), and AcOH (2 mL) was heated at 140 °C for 2 h under microwave irradiation. The mixture was quenched with water and extracted with EtOAc. The organic layer was separated, washed with sat. aq. NaHCO₃ and water, dried over Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with MeOH in EtOAc). The obtained solid was triturated in diisopropyl ether and EtOAc and collected by filtration to give 1-(cyclopentylmethyl)-2,2-dimethyl-7-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridin-1-yl)-2,3-dihydroquinazolin-4(1*H*)-one (20 mg, 30%) as a white solid. ¹H NMR (DMSO-*d*₆) δ: 1.19–1.33 (2H, m), 1.37–1.78 (12H, m), 2.11–2.27 (1H, m), 3.13 (2H, d, *J* = 6.8 Hz), 6.88–6.97 (2H, m), 7.03 (1H, dd, *J* = 7.9, 5.3 Hz), 7.42 (1H, dd, *J* = 7.7, 1.3 Hz), 7.84 (1H, d, *J* = 8.7 Hz), 8.00 (1H, dd, *J* = 4.9, 1.1 Hz), 8.21 (1H, s), 11.88 (1H, br s). LC-MS (ESI) *m/z* = 392.3 [M+H]⁺.

Compounds **6** and **7** were prepared in a manner similar to that described for **9**.

5.1.3.4. 1-(Cyclopropylmethyl)-2,2-dimethyl-7-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridin-1-yl)-2,3-dihydroquinazolin-4(1*H*)-one (**6**)

Compound **6** was obtained (90%) as a white solid. ¹H NMR (DMSO-*d*₆) δ: 0.30 (2H, q, *J* = 4.8 Hz), 0.47–0.55 (2H, m), 0.99–1.12 (1H, m), 1.46 (6H, s), 3.19 (2H, d, *J* = 5.7 Hz), 6.91 (1H, dd, *J* = 8.1, 1.7 Hz), 6.98–7.07 (2H, m), 7.42 (1H, dd, *J* = 7.9, 1.1 Hz), 7.85 (1H, d, *J* = 8.3 Hz), 7.99 (1H, dd, *J* = 5.1, 1.3 Hz), 8.19 (1H, s), 11.89 (1H, br s). LC-MS (ESI) *m/z* = 364.3 [M+H]⁺. 93% purity.

5.1.3.5. 2,2-Dimethyl-1-(2-methylpropyl)-7-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridin-1-yl)-2,3-dihydroquinazolin-4(1*H*)-one (**7**)

Compound **7** was obtained (15%) as a white solid. ¹H NMR (DMSO-*d*₆) δ: 0.93 (6H, d, *J* = 6.8 Hz), 1.43 (6H, s), 1.85–1.98 (1H, m), 3.00 (2H, d, *J* = 7.2 Hz), 6.86–6.96 (2H, m), 7.05 (1H, dd, *J* = 7.7, 5.1 Hz), 7.41 (1H, dd, *J* = 7.9, 1.1 Hz), 7.85 (1H, d, *J* = 7.9 Hz), 8.00 (1H, dd, *J* = 5.3, 1.1 Hz), 8.23 (1H, s), 11.88 (1H, s). LC-MS (ESI) *m/z* = 366.3 [M+H]⁺.

5.2. Molecular docking procedures

The X-ray crystal structure of PKCθ bound to staurosporine (PDB code: 1XJD) was used as the target structure for docking. The PKCθ protein for docking was prepared by using the Protein Preparation Wizard, pre-processed and heterostate for co-crystallized ligand was generated using Epik. The protonation state and optimized H-bonds of the protein side chains were assigned using Protassign. Energy minimizations were performed using the OPLS_2005 force field. The ligand was prepared by using the LigPrep¹⁶ utility of the Schrodinger Suite with the default parameters. Conformational searching was conducted using MacroModel¹⁷ with the default parameters and the derived conformational structures were docked into the ATP binding-site of PKCθ using Glide.¹⁸ The docking poses were evaluated by the empirical scoring function, GlideScore.

5.3. Kinase sequence alignments

Protein kinase sequences were obtained from kinome-render¹⁹ and aligned using Clustal Omega²⁰. From the generated alignment of the kinase sequences, information about the type and number of the amino acids corresponding to the Thr442 residue of PKCθ was obtained. Atypical kinases and kinases without amino acids corresponding to PKCθ Thr442 were removed.

5.4. Enzyme assays for PKC subfamily

PKCθ, α, β, δ, and ζ were expressed as N-terminal FLAG fusion proteins using a baculovirus expression system. Assays for each PKC subtype were performed in 384-well plates using the Lanthascreen system (Invitrogen) at room temperature. Enzyme reactions were performed in 20 mM Tris-HCl, at pH 7.5, containing 0.05% bovine serum albumin, 1 mM dithiothreitol,

0.1 mM CaCl₂, 5 mM Mg(OAc)₂, 0.01% Tween-20, 0.4 μM Fluorescein-PKC substrate (Invitrogen), 10% PKC activator (Millipore), and optimized concentrations of enzyme and ATP. Prior to the enzyme reaction, the compound and enzyme were incubated for 5 min at room temperature. The reactions were initiated by the addition of ATP. After 60 min, the reactions were stopped by the addition of the same volume of TR-FRET dilution buffer (Invitrogen) containing 40 mM EDTA and Lanthascreen Tb-pSer (PKC) antibody (1/20000 volume of the reaction mixture) (Invitrogen). The plates were incubated for 30 min and then read using an Envision Multilabel reader (PerkinElmer). The reaction conditions were optimized for each PKC subtype: PKCθ (1 pM enzyme, 20 μM ATP); PKCα (0.03 pM enzyme, 20 μM ATP); PKCβ (0.03 pM enzyme, 20 μM ATP); PKCδ (1 pM enzyme, 20 μM ATP); PKCζ (3 pM enzyme, 5 μM ATP).

5.5. Cell-based assay

Jurkat cells were maintained in RPMI 1640 medium containing 10% FCS, 100 units/mL penicillin and 100 μg/mL streptomycin. 96-Well plates were coated with 100 μL of PBS containing 500 μg/mL anti-CD3 antibody (R&D systems) and incubated overnight at 4 °C. The coated plates were washed twice with PBS before Jurkat cells, transfected with pHTS-NFκB (Biomyx) by electroporation, were added to the plate. Then compound **14** diluted in RPMI 1640 medium were added to the cells, and the cells were stimulated by addition of 0.5 μg/mL anti-CD28 antibody (Pharmingen). The plates were incubated overnight at 37 °C before the same volume of Bright-Glo (Invitrogen) was added. After a few minutes incubation, luminescence was measured using an Envision Multilabel reader (PerkinElmer).

5.6. Kinase selectivity profile

5.6.1. Serine/threonine kinases and PI3Kα profiling

Assays for serine/threonine kinases using radio labeled [γ-³³P]ATP (GE Healthcare, Piscataway, NJ) were performed in 96-well plates. Mitogen-activated protein kinase p38 alpha (p38α), extracellular signal-regulated kinase 1 (ERK1), polo-like kinase 1 (PLK1), apoptosis signal-regulating kinase 1 (ASK1), and MAPKAPK2 were expressed as N-terminal FLAG-tagged proteins using a baculovirus expression system. Aurora-B was expressed as an N-terminal 6xHis-tagged protein using a baculovirus expression system. N-terminal 6xHis-tagged PI3Kα co-expressed with p85α was expressed using a baculovirus expression system.

MEK1 was expressed as an N-terminal GST fusion protein using a freestyle293 (Invitrogen Life Technologies) expression system. Casein kinase 1 delta (CK1δ) was expressed as an N-terminal GST fusion protein using an *E. coli* expression system. Checkpoint kinase 1 (CHK1) and glycogen synthase kinase 3β (GSK-3β) were expressed as N-terminal GST fusion proteins using a baculovirus expression system. CDK2/CycA was expressed as C-terminal 6xHis-tagged CDK2 and N-terminal GST-tagged cyclin A proteins using a baculovirus expression system. Interleukin-1 receptor associated kinase 4 (IRAK-4), Rho-associated, coiled-coil-containing protein kinase 1 (ROCK1), and cell division cycle 7 (CDC7) were purchased from Carma Biosciences (Japan).

The reaction conditions were optimized for each kinase: p38α (100 ng/well of enzyme, 1 μg/well of myelin basic protein (MBP) (Wako Pure Chemical Ind.), 0.1 μCi/well of [γ-³³P]ATP,

60 min reaction at 30 °C); ERK1 (100 ng/well of enzyme, 2 μg/well of MBP, 0.1 μCi/well of [γ-³³P]ATP, 60 min reaction at 30 °C); MEK1 (100 ng/well of enzyme, 0.3 μg/well of GST-ERK1 (K71A) 0.2 μCi/well of [γ-³³P]ATP, 20 min reaction at room temperature); Aurora-B (50 ng/well of enzyme, 30 μM of Aurora substrate peptide, 0.2 μCi/well of [γ-³³P]ATP, 60 min reaction at room temperature); CDK2/CycA (1.8 ng/well of enzyme, 1 μg/well of Histone H1, 0.2 μCi/well of [γ-³³P]ATP, 20 min reaction at room temperature); CK1δ (120 ng/well of enzyme, 2.4 μM of CK1tide (Millipore Corp.), 0.2 μCi/well of [γ-³³P]ATP, 20 min reaction at room temperature); CHK1 (30 ng/well of enzyme, 25 μM of CHKtide (Millipore Corp.), 0.2 μCi/well of [γ-³³P]ATP, 10 min reaction at room temperature); GSK-3β (25 ng/well of enzyme, 0.2 μg/well of GSK3 Substrate Peptide (Millipore Corp.), 0.1 μCi/well of [γ-³³P]ATP, 30 min reaction at room temperature). ASK1 (25 ng/well of enzyme, 2 μg/well of MBP (Wako Pure Chemical Ind.), 0.1 μCi/well of [γ-³³P]ATP, 60 min at room temperature); MAPKAPK2 (300 ng/well of enzyme, 0.5 μM of KKLNRRLSVA-NH₂, 0.1 μCi/well of [γ-³³P]ATP, 10 min at room temperature); ROCK1 (164 ng/well of enzyme, 0.5 μM of Long S6 peptide (Millipore Corp.), 0.2 μCi/well of [γ-³³P]ATP, 10 min at room temperature); PLK1 (25 ng/well of enzyme, 3 μg/well of α-casein (Sigma Chemical Co.), 0.2 μCi/well of [γ-³³P]ATP, 60 min at room temperature); IRAK4 (27.7 ng/well of enzyme, 1 μM of MBP (Wako Pure Chemical Ind.), 0.2 μCi/well of [γ-³³P]ATP, 60 min at room temperature); CDC7 (3.1 ng/well of enzyme, 1 μg of MCM2), 0.2 μCi/well of [γ-³³P]ATP, 60 min at room temperature).

Prior to the kinase reactions, the compounds and enzymes were incubated for 5 min at the reaction temperatures described above. The kinase reactions were initiated by the addition of ATP. After the reaction periods described above, the reactions were terminated by the addition of 10% trichloroacetic acid (final concentration). The [γ-³³P]-phosphorylated proteins were filtered in a Harvest Plate (Millipore Corp.) with a Cell Harvester (PerkinElmer) and then free [γ-³³P]ATP was washed out using 3% phosphoric acid. The plates were dried, and 40 μL of MicroScint™ 0 (PerkinElmer) was added. The radioactivity was counted using a TopCount scintillation counter (PerkinElmer).

To assay for PI3Kα, the Phospholipid FlashPlate™ method was used. Plates were coated with 5 μM L-α-phosphatidyl-D-myo-inositol 4,5-diphosphate (diC16) and incubated overnight at room temperature. The reaction conditions were optimized for PI3Kα (80 ng/well of enzyme, fixed diC16 on the plate, 0.15 μCi/well of [γ-³³P]ATP, 60 min at room temperature).

5.6.2. Tyrosine kinase profiling

The cytoplasmic domain of vascular endothelial growth factor receptor 2 (VEGFR2) and focal adhesion kinase (FAK) were expressed as an N-terminal FLAG-tagged proteins using the baculovirus expression system. These expressed kinase proteins were purified using an anti-FLAG M2 affinity gel (Sigma-Aldrich). c-Met, Src, epidermal growth factor receptor (EGFR), and insulin receptor kinase (IRK) were purchased from Millipore Corp. Janus kinase 1 (JAK1), TrkA, and EphA5 were purchased from Carma Biosciences (Japan).

Assays for tyrosine kinases using anti-phosphotyrosine antibodies were performed at room temperature in 384-well plates using the Alphascreen® system (PerkinElmer). Enzyme reactions were performed in 50 mM Tris-HCl, at pH 7.5, containing 5 mM MnCl₂, 5 mM MgCl₂, 0.01% Tween-20, 2 mM dithiothreitol, and 0.1 μg/ml biotinylated poly-GluTyr (4:1) The

optimized concentrations of enzyme and ATP are described below.

Prior to the kinase reactions, the compounds and enzymes were incubated for 5 min at room temperature. The reactions were initiated by the addition of ATP. After the reaction period (described below), the reactions were stopped by the addition of 25 μ L of 100 mM EDTA, 10 μ g/ml Alphascreen streptavidine donor beads, and 10 μ g/mL acceptor beads (described below) in 62.5 mM HEPES, pH 7.4, 250 mM NaCl, and 0.1% BSA. The plates were incubated in the dark for more than 12 h and then read using an EnVision 2102 Multilabel Reader (PerkinElmer). A well containing a substrate and an enzyme without a compound was used as a total control reaction. The reaction conditions for these kinases were optimized for each kinase: VEGFR2 (19 ng/ml of enzyme, 10 μ M ATP, 10 min reaction, PY-100 conjugated acceptor beads (PY-100)); c-Met (1 ng/ml of enzyme, 2 μ M ATP, 10 min reaction, PT66); Src (0.33 ng/ml of enzyme, 2 μ M ATP, 10 min reaction, PY-100); EGFR (100 ng/ml of enzyme, 1 μ M ATP, 10 min reaction, PT66); IRK (100 ng/ml of enzyme, 10 μ M ATP, 60 min reaction, PT66); FAK (30 ng/ml of enzyme, 2 μ M ATP, 20 min reaction, PT66); EphA (1 ng/ml of enzyme, 2 μ M ATP, 10 min reaction, PY-100); JAK1 (100 ng/ml of enzyme, 0.2 μ M ATP, 10 min reaction, PT66); TrkA (0.1 ng/ml of enzyme, 1 μ M ATP, 60 min reaction, PY-100); EphA5A (1 ng/ml of enzyme, 2 μ M ATP, 10 min reaction, PY-100).

5.7. Human whole blood IL-2 assay

Heparinized human whole blood was obtained from healthy volunteers and diluted two-fold with RPMI1640 medium supplemented with penicillin, streptomycin and 10% fetal calf serum. Then, 270 μ L aliquots of diluted whole blood were added to a 96-well plate and stimulated with 30 μ L/well of Dynabeads® Human T-Activator CD3/CD28 (Life Technologies) and compound **14** at 37 °C in a 5% CO₂ incubator. After 24 h of incubation, 120 μ L of the supernatant was harvested from each well, and the IL-2 concentration was measured using ELISA (R&D systems, Human IL-2 Duo set). ELISA was performed according to the manufacturer's instructions.

5.8. Mouse IL-2 PD assay

Female BALB/c mice (8 weeks old, Charles River, Japan) were administered 10 mg/kg or 100 mg/kg of either compound **14** or vehicle (0.5% methylcellulose solution) orally. One hour after oral administration of compound **14**, the mice were injected with 5 μ g of anti-CD3 ϵ (hamster anti-mouse IgG clone 145-2C11, Bio X Cell) intraperitoneally. Three hours after injection, blood samples were obtained by retro-orbital venous plexus puncture and collected in tubes with heparin. The plasma concentration of mouse IL-2 was measured using a Mouse IL-2 Quantikine ELISA Kit (R&D Systems) according to the manufacturer's instructions. Six mice were used for each group.

5.9. Protein expression and purification.

The cDNA encoding the PKC θ kinase domain (residues 361-706) was subcloned into the pFastBac1 vector, with the reading frame engineered to harbor two mutations (I381E, T538E) plus a 6xHis tag at the C-terminus. Recombinant virus was generated using the Bac-to-Bac baculovirus expression system (Invitrogen). For kinase domain protein expression, two

liters of virus-infected Hi5 cells were grown at 27 °C for 64 h in ESF921 medium (Expression Systems) supplemented with 30 μ M of compound **10**. Cells were homogenized in 600 mL of buffer containing 25 mM Tris pH 7.9, 1 M NaCl, 20 mM imidazole, 0.25 mM TCEP, and protease inhibitor cocktail (Roche), plus 5 μ M of compound **10**. The lysate supernatant was loaded onto a column packed with a nickel-chelating resin (ProBond, Invitrogen) and eluted with buffer containing 300 mM imidazole. The protein was then further purified using a Superdex 75 column (GE Healthcare) in buffer containing 25 mM Tris pH 8.0, 200 mM NaCl, 1 mM NaF, and 5 mM TCEP. The protein was concentrated with 0.1 mM of compound **10** to a final concentration of 15 mg/mL before crystallization.

5.10. Crystallization, diffraction data collection, and structure determination.

Crystallization was performed at 20 °C using the hanging drop vapor diffusion method. Crystallization drops contained PKC θ protein solution at 15 mg/mL preincubated with 0.1 mM of compound **10** (described above), and reservoir solution [(0.1 M Hepes pH 7, 0.2 M lithium citrate, and 18% PEG M.W. 3,350, 1% hexanediol (Buffers and salt solutions were prepared on-site using chemicals purchased from SIGMA, solutions of crystallization grade PEG, ethylene glycol, and hexanediol, were from Hampton Research)]. Crystals were cryo-protected by transferring them into a reservoir solution containing 30% ethylene glycol and flash-cooling in liquid N. Diffraction data were collected at the Advanced Light Source Beamline 5.0.3 (Lawrence Berkley's National Laboratory, Berkley, CA). Diffraction data were processed with HKL2000.²¹ The structure was determined by molecular replacement with Phaser²² using the coordinates of PKC θ (PDB code: 1XJD) as a search model. The graphics program COOT²³ was used for model building, and refinement was performed with REFMAC5.²⁴ Phaser and REFMAC5 are distributed as part of CCP4.²⁵

Structure	PKC θ compound 10
PDB entry	5F9E
Data Collection	
Resolution (Å)	50.00–2.00
(Highest shell, Å)	2.03–2.00
Space Group	P2 ₁ 2 ₁ 2 ₁
Unit Cell Dimensions:	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	75.60, 76.79, 149.3
α , β , γ (°)	90, 90, 90
Unique reflections	59517
Overall Statistics (Highest Shell)	
R _{sym} (%)	5.5(84.5)
I/ σ _I	29.5(1.6)
Data completeness (%)	97.4(81.1)
Redundancy	6.9(4.4)
Refinement	
Reflections	54,956
R _{cryst} (%)	20.3
R _{free} (%)	23.8
R.m.s deviations:	

bond lengths (Å)	0.009
bond angles (°)	1.422

5.11. Metabolic clearance assay

In vitro oxidative metabolic studies of the tested compounds were carried out using hepatic microsomes obtained from mice. The reaction mixture, with a final volume of 0.05 mL, consisted of 0.2 mg/mL hepatic microsome in 50 mM $\text{KH}_2\text{PO}_4\text{--K}_2\text{HPO}_4$ phosphate buffer (pH 7.4) and 1 μM of the test compound. The reaction was initiated by the addition of an NADPH-generating system containing 50 mM MgCl_2 , 50 mM glucose 6-phosphate, 5 mM $\beta\text{-NADP}^+$, and 15 unit/mL glucose 6-phosphate dehydrogenase at 10% volume of the reaction mixture. After the addition of the NADPH-generating system, the mixture was incubated at 37 °C for 0 min, 15 min, and 30 min. The reaction was terminated by the addition of an equivalent volume of CH_3CN . After the samples were mixed and centrifuged, the supernatant fractions were analyzed using liquid chromatography tandem mass spectrometry. For metabolic clearance determinations, chromatograms were analyzed for parent compound disappearance rates from the reaction mixtures. All incubations were made in duplicate.

Acknowledgments

We would like to acknowledge Dr. Takashi Ichikawa, Dr. Ikumi Chisaki, Dr. Nao Morishita, and Dr. Noboru Tsuchimori for helpful discussion during the preparation of the manuscript. We also thank the staff of the Berkeley Center for Structural Biology, Lawrence Berkeley National Laboratory, which operates Advanced Light Source Beamline 5.0.3. The Berkeley Center for Structural Biology is supported in part by the National Institutes of Health and National Institute of General Medical Sciences. The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences of the US Department of Energy under contract number DE-AC02-05CH11231.

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- Detailed statement of residues (Val=238 kinases, Ile=101 kinases, Leu=42 kinases, Thr=21 kinases, Ala=9 kinases, Cys=2 kinases, His=2 kinases, Met=2 kinases, Pro=2 kinases, Lys=1 kinase, Asn=1 kinases, Gln=1 kinases, Ser=1 kinases).
- >30% inhibition at 1 μM : 0/26 kinases (c-Met=7%, FAK=2%, IRAK4=5%, TrkA=18%, MEK1=2%, ASK1=6%, CK1 δ =2%, PKA=3%, AuroraB=12%, ROCK1=9%, MAPKAPK2=1%, ERK1=4%, CDK2=7%, p38 α =16%, GSK3 β =27%, CDC7=2%, PI3K α =2%).
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Discovery and optimization of 1,7-disubstituted-2,2-dimethyl-2,3-dihydroquinazolin-4(1*H*)-ones as potent and selective PKC θ inhibitors

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