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C-5 substituted heteroaryl-3-pyridinecarbonitriles as PKCθ inhibitors: Part II

Amar S. Prashad [a,*], Daniel Wang [a], Joan Subrath [a], Biqi Wu [a], Melissa Lin [a], Mei-Yi Zhang [b], Natasha Kagan [b], Julie Lee [c], Xiaoke Yang [c], Agnes Brennan [c], Divya Chaudhary [c], Xin Xu [d], Louis Leung [e], Jack Wang [e], Diane H. Boschelli [a]

[a] Wyeth Research, Chemical Sciences, 401 N. Middletown Road, Pearl River, NY 10965, United States
[b] Wyeth Research, Chemical Sciences, CN 8000, Princeton, NJ 08543, United States
[c] Wyeth Research, Inflammation, 200 Cambridge Park Drive, Cambridge, MA 02140, United States
[d] Wyeth Research, Drug Safety and Metabolism, 1 Burtt Road, Andover, MA 01810, United States
[e] Wyeth Research, Drug Safety and Metabolism, 500 Arcola Road, Collegeville, PA 19426, United States

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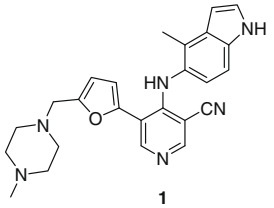
ABSTRACT

We previously reported that a 3-pyridinecarbonitrile analog with a furan substituent at C-5 and a 4-methylindol-5-ylamino substituent at C-4, **1**, was a potent inhibitor of PKCθ (IC_{50} = 4.5 nM). Replacement of the C-5 furan ring of **1** with bicyclic heteroaryl rings, led to compounds with significantly improved potency against PKCθ. Analog **6b** with a 4-methylindol-5-ylamino group at C-4 and a 5-[(4-methylpiperazin-1-yl)methyl]-1-benzofuran-2-yl group at C-5 had an IC_{50} value of 0.28 nM for the inhibition of PKCθ.

Protein kinases are critical regulators of cellular processes in normal and disease states. The protein kinase C family is a group of serine threonine kinases that share sequence and structural homology.[1] The classical PKC isoforms, α, βIβII, and γ, require the second messengers calcium and diacylglycerol (DAG). The novel isoforms, δ, ε, η, and θ, require DAG but not calcium. The atypical isoforms, ζ and λ, do not require either calcium or diacylglycerol.

PKCθ is found predominantly in T cells and skeletal muscle. It has been demonstrated that PKCθ plays an integral role in the activation and survival of T cells.[2–4] Validation studies using PKCθ knock-out (KO) mice have established therapeutic utility in diseases such as multiple sclerosis, arthritis, asthma, inflammatory bowel disease, and transplant rejection.[5] However, despite this diverse therapeutic potential there is only scant published work on selective small molecules targeting PKCθ. Boehringer Ingelheim has disclosed a series of 2,4-diaminopyrimidines,[6,7] and we previously reported that thieno[2,3-*b*]pyridine-5-carbonitriles[8,9] and 3-pyridinecarbonitriles[10–12] can be used as templates for inhibitors of this kinase. Regarding the 3-pyridinecarbonitriles, we earlier demonstrated that a 4-methyl-5-indolyl amino group at the 4-position was optimum for potency against PKCθ and selectivity against PKCδ.[12] We used PKCδ as our primary counter screen since PKCδ KO mice were found to be susceptible to the development of autoimmune diseases.[13,14]

Compound **1** displayed reasonable potency against PKCθ kinase activity with an IC_{50} value of 4.5 nM and had 10-fold selectivity against PKCδ, however it suffered from poor metabolic stability.[15,16] In an effort to improve on the stability of **1**, the C-5 furan ring was replaced with various bicyclic heteroaryls while retaining the optimal 4-methylindol-5-ylamino substituent at the 4-position. A key intermediate **2** (Scheme 1) that facilitated the synthesis of these compounds has been previously reported.[12] Using this intermediate many analogs of the desired bicyclic heteroaryl series were prepared in an expedient manner.



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Thus advanced intermediate **2** was subjected to typical Suzuki protocol using benzofuran-2-boronic acid and benzothiophene-2-boronic acid to prepare compounds **3a** and **4a**, respectively (Scheme 1). In order to add water solubilizing groups to the bicyclic heteroaryls, the 5-formyl analogs of benzofuran and benzothiophene were prepared. The 5-formyl analog of benzofuran, **3b**, was prepared via a Stille coupling of **2** and **5**, 2-(tributylstannyl)-5-benzofurancarboxaldehyde (Scheme 1). The stannane was prepared by a two-step sequence involving DIBAL-H reduction of

* Corresponding author. Tel.: +1 845 602 5310; fax: +1 845 602 5561.
E-mail address: prashaa@wyeth.com (A.S. Prashad).

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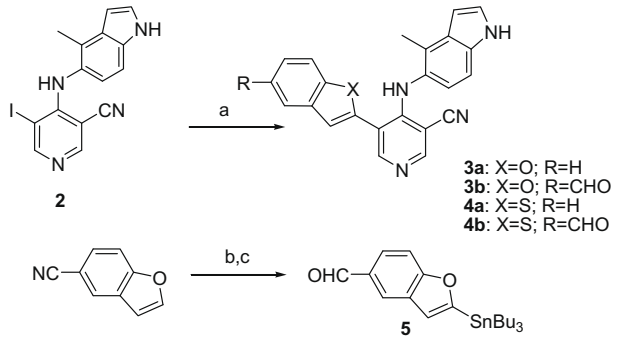
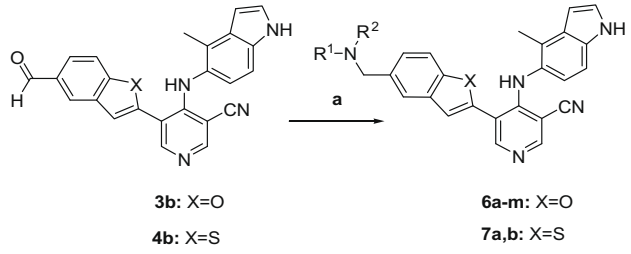


Scheme 1. (a) Reagents and conditions: for **3a**: 2.0 equiv benzofuran-2-boronic acid, 0.05 equiv $Pd(PPh_3)_4$, aq Na_2CO_3, DME, 80 °C, 2 h; for **3b**: 1.5 equiv **5**, 0.05 equiv $Pd(PPh_3)_4$, DMF, 80 °C, 2 h; for **4a**: 1.1 equiv benzothiophene-2-boronic acid, 0.05 equiv $Pd(PPh_3)_4$, aq Na_2CO_3, DME, 100 °C, 12 h; for **4b**: 1.1 equiv 2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzothiophene-5-carboxaldehyde, 0.05 equiv $Pd(PPh_3)_4$, aq Na_2CO_3, DME, 100 °C, 12 h; (b) 1.2 equiv DIBAL-H, CH_2Cl_2, −15 °C, 10 min; (c) 3.3 equiv *n*BuLi, 1.1 equiv *N*-Me-piperazine, 2.2 equiv Bu_3SnCl, THF, 0 °C, 6h.



Scheme 2. (a) Reagents and conditions: for **6a–i**, **k**, **m**: 3.0 equiv R^1R^2NH, 5.5 equiv AcOH, 3.0 equiv $Na(OAc)_3BH$, THF/EtOH, rt, 12 h; for **6j**, **l**: 4.0 equiv R^1R^2NH, 4.0 equiv AcOH, 5.0 equiv $Na(OAc)_3BH$, CH_2Cl_2/NMP, rt, 12 h; for **7a**: 2.0 equiv R^1R^2NH, 1.2 equiv AcOH, 1.3 equiv $NaCNBH_3$, EtOH, rt, 12 h; for **7b**: 5.0 equiv R^1R^2NH, 5.0 equiv AcOH, 5.0 equiv $Na(OAc)_3BH$, THF, rt, 12 h.

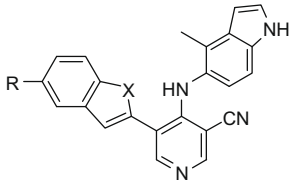
1-benzofuran-5-carbonitrile to give the corresponding aldehyde which upon treatment with *n*BuLi/*N*-Me-piperazine and tributyltin chloride resulted in intermediate **5**. The 5-formyl analog of benzothiophene, **4b**, was prepared in a similar manner to **3a** and **4a** using the corresponding boronic acid, pinacol ester as shown in Scheme 1. The aldehydes **3b** and **4b** were then subjected to a reductive amination protocol, as shown in Scheme 2, using various amines to provide analogs shown in Tables 1 and 2.

Table 1 shows three exact comparisons between benzofuran and benzothiophene analogs. In all three cases the benzofuran analogs, exemplified by **3a**, **6a**, and **6b** (IC_{50} = 1.7, 1.3, 0.28 nM, respectively) showed greater potency against PKCθ kinase activity than their benzothiophene counterparts exemplified by **4a**, **7a**, and **7b** (IC_{50} = 23, 2.5, 0.67 nM, respectively). This increase in potency was accompanied by enhanced selectivity over PKCδ in all three of the examples mentioned above. For example, **6b** (benzofuran series) had an IC_{50} value of 17 nM for PKCδ inhibition which corresponds to a 61-fold selectivity for PKCθ over PKCδ, whereas for **7b** (benzothiophene series) the IC_{50} value was 9.9 nM which corresponds to a 15-fold selectivity for PKCθ over PKCδ. Since the benzofuran series exhibited superior potency for PKCθ inhibitory activity and enhanced selectivity over PKCδ compared to the benzothiophene series, further modifications were confined to the benzofuran series.

Table 2 shows various benzofuran analogs, **6a–m** where the amine in the reductive amination protocol was varied (see Scheme 2). Compounds **6a–m** with various types of amine substituents displayed generally good potency against PKC theta, ranging from an IC_{50} value of 0.28 nM for **6b** to 15 nM for **6l**. Many of the compounds with secondary amine substituents, such as **6a, b, d** were significantly more selective for PKCθ over PKCδ than those compounds with primary amine substituents, whereas others such as **6c,e** exhibited similar selectivity. In fact, the most selective compound with a secondary amine was **6b** having a selectivity of 61-fold for PKCθ over PKCδ. The most selective compound with a primary amine substituent was **6i** having a selectivity of 10-fold for PKCθ over PKCδ.

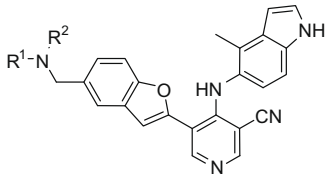
At this juncture, the *N*-Me-piperazine analog, **6b**, exhibited the best overall potency and selectivity profile and hence an expanded SAR (structure–activity relationships) analysis was conducted on this piperazine moiety. Table 2 shows SAR on analog **6b** where the substituent on the distal *N* of the piperazine ring was varied. These compounds were prepared (Scheme 2) using the corresponding amine in the reductive amination protocol with **3b**. In going from a methyl to an ethyl substituent; that is, from **6b** to **6j**, the PKCθ IC_{50} value decreased only slightly, from 0.28 nM in **6b** to 0.48 nM in **6j**, while the PKCδ inhibitory activity was virtually unchanged (IC_{50} = 17 and 16 nM for **6b** and **6j**, respectively). Analog **6k**, with a 2-dimethylamino(ethyl) substituent on the *N* of the piperazine ring, had about 10-fold reduced potency for PKCθ inhibition relative to **6b**, and its selectivity for PKCθ over PKCδ was significantly lower. The methyl sulfone, **6l** (PKCθ IC_{50} = 15 nM), and 2-pyridyl, **6m** (PKCθ IC_{50} = 13 nM), analogs however, exhibited both diminished activity and selectivity relative to **6b**. The methyl substituted analog, **6b**, stood out as the most potent and selective compound of all the analogs not only in the piperazine subseries but also in the entire C-5 benzofuran series.

Table 1
PKCθ and PKCδ inhibitory activity of benzofurans and benzothiophenes

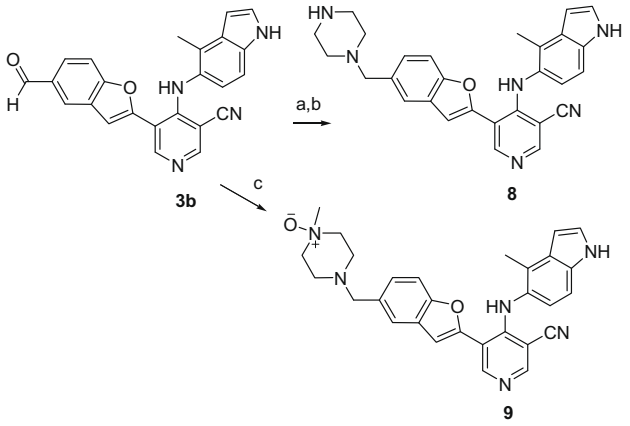


Ex	X	R	PKCθ[21] IC_{50} nM	PKCδ[21] IC_{50} nM	PKCδ/PKCθ
3a	O	H	1.7	57	33
4a	S	H	23	154	7
6a	O	CH_2–piperidine	1.3	25	19
7a	S	CH_2–piperidine	2.5	5.2	2
6b	O	CH_2–N–Me–piperazine	0.28	17	61
7b	S	CH_2–N–Me–piperazine	0.67	9.9	15

Table 2
PKCθ and PKCδ inhibitory activity of benzofuran analogs with various substitutions at C-5



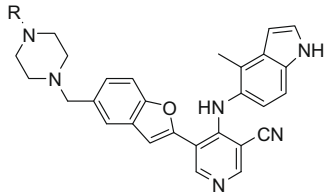
Ex	NR^1R^2	PKCθ[21] (IC_{50} nM)	PKCδ[21] (IC_{50} nM)	PKCδ/PKCθ
6a	Piperidine	1.3	25	19
6b	*N*-Me-piperazine	0.28	17	61
6c	Me_2N	6.4	44	7
6d	Pyrrolidine	2.9	43	15
6e	Morpholine	13	121	9
6f	NH-cyclohexyl	1.4	10	7
6g	$NH(CH_2)_2OH$	1.6	13	8
6h	$NH(CH_2)_3OH$	2.3	21	9
6i	$NHCH_2CH(OH)CH_2OH$	1.5	16	10
6j	*N*-Et-piperazine	0.48	16	33
6k	*N*-$(CH_2)_2NMe_2$-piperazine	2.4	20	8
6l	*N*-SO_2Me-piperazine	15	138	9
6m	*N*-2-Pyridyl-piperazine	13	149	12



Scheme 3. Reagents and conditions: (a) 4.0 equiv *N*-Boc-piperazine, 4.0 equiv AcOH, 5.0 equiv $Na(OAc)_3BH$, CH_2Cl_2/NMP, rt, 12 h; (b) 10% trifluoroacetic acid/CH_2Cl_2, rt, 2h; (c) 5.0 equiv 1-methylpiperazine-1-oxide, 4.0 equiv AcOH, 5.0 equiv $Na(OAc)_3BH$, CH_2Cl_2/NMP, rt, 12 h.

Incubation of **6b** with rat, mouse, and human liver microsomes fortified with cytosol provided half-lives of 27, 17, and 26 min, respectively. Reduced half-lives of 12 and 10 min were observed with dog and monkey microsomes. Metabolite ID by LC/MS/MS identified the major metabolite of **6b** in rats and dogs to be a single oxidation product (M+16). The major metabolite in monkey and human microsomes was loss of the methyl group from the *N*-Me-piperazine to give **8**. This desmethyl analog was seen as a minor metabolite in the rat and dog microsome studies. To identify the site of the oxidative metabolism, a large scale incubation of **6b** with rat microsomes was carried out to provide enough material for an NMR study. Comparison of the proton spectra of the parent and metabolite revealed the most significant difference to be the chemical shifts of the *N*-Me of the piperazine and of the adjacent methylene protons. The *N*-Me signal of **6b** was observed at 2.20 ppm while the corresponding signal for the metabolite was at 3.16 ppm, suggesting that the major M+16 metabolite was oxidation of the nitrogen substituted with the Me group to provide **9**. Accordingly these analogs were prepared in order to assess their PKCθ and PKCδ inhibitory activities (Scheme 3). In the preparation of **8**, a similar reductive amination protocol as in Scheme 2 was used where the amine was *N*-Boc piperazine. After the reductive amination, the *N*-Boc group was cleaved with trifluoroacetic acid to give **8**. In the preparation of **9**, 1-methylpiperazine-1-oxide[17] was subjected to a reductive amination protocol with **3b** to provide **9**. Both of these metabolites displayed about 20-fold decreased potency against PKCθ activity compared to **6b** (Table 3). However, their selectivities over PKCδ were quite different with **8** having a selectivity of less than onefold while that of **9** was 18-fold, both being significantly lower, relative to that of **6b** (61-fold).

Table 3
Enzyme and cell activity of **6b** and its metabolites



Ex	R	PKCθ[21] (IC_{50} nM)	PKCδ[21] (IC_{50} nM)	WT T-cell IL2[21] (IC_{50} nM)	KO T-cell IL2[21] (IC_{50} nM)
6b	Me	0.28	17	21	880
8	H	5.3	4.3	16	1500
9	Me(O)	6.1	110	360	>10,000

The cellular activities of **6b** and its metabolites were evaluated in assays using murine T cells stimulated with anti-CD3 and anti-CD28 to induce IL-2 expression.[10] With T cells from WT mice, **6b**, blocked the production of IL-2 with an IC_{50} value of 21 nM, a four-fold increase in activity compared to that of **1** (IC_{50} = 86 nM[15,16]). Reduced activity was seen in a corresponding assay with T cells isolated from PKCθ KO mice where **6b** had an IC_{50} value of 0.88 μM. This sub-micromolar IC_{50} seen in **6b** for PKCθ deficient cells may be due, in part, to the inhibition of other kinases that are involved in IL-2 production. The desmethyl metabolite of **6b**; that is, **8** had similar WT T cell IL-2 activity (IC_{50} = 16 nM) as **6b** whereas the N-oxide metabolite, **9**, had greatly reduced activity (IC_{50} = 360 nM). It is unclear why there is a lack of correlation between the enzyme and cell-based assays for metabolite **8**. The permeability of **8** relative to **6b** does not explain this.

Analog **6b** was profiled against additional PKC family members. While **6b** only weakly inhibited PKCβ (IC_{50} = 0.8 μM), a classical isoform, more potent inhibition of PKCε and PKCη, two novel PKCs was observed, with **6b** having IC_{50} values of 2.5 and 33 nM, respectively. No inhibition of PKCξ, an atypical isoform, was observed (IC_{50} >100 μM). Some additional kinase profiling was done for **6b**. Compound **6b** displayed exquisite selectivity for PKCθ over other kinases including MK2, p38, IKK, PDGFR, and ROCK1 having IC_{50} values of greater than 9 μM. Relatively lesser selectivity was observed for Lyn and Lck with **6b** having IC_{50} values of 0.73 and 0.26 μM, respectively. However, this still represents an impressive selectivity of approximately 1000–2000-fold of **6b** for PKCθ inhibition over members of the Src kinase family.

In pharmaceutical profiling assays, **6b** had a good permeability of 3.96×10^{-6} cm/s in a PAMPA format, with excellent solubility at pH 4.5 (>100 μg/mL). In assays looking at the reversible inhibition of cytochrome P450 activity in human liver microsomes, **6b** had IC_{50}s of greater than 10 μM (CYPs 1A2, 2A6, 2C9, 2C19, 2D6, and 3A4). Furthermore this compound was also a weak hERG inhibitor having an IC_{50} value of greater than 30 μM.

Following a single iv dose of 2 mg/kg to male Sprague Dawley rats, **6b** had a clearance of 55 mL/min/kg and a volume of distribution of 7.3 L/kg. Following a single oral dose of 10 mg/kg, **6b** had a C_{max} of 108 ng/mL and an AUC of 1140 ng h/mL with an oral bioavailability of 35%. Both the metabolites of **6b** were observed in the plasma samples from these pharmacokinetic studies. Interestingly, the desmethyl analog **8** was the major metabolite being present at about 20% with the N-oxide **9** being present at about 8%.

In conclusion, **6b** exhibited an overall highly desirable profile and was taken on into further cellular and in vivo studies. Some of these results have been recently presented and will be the subject of a future publication.[18–20]

Acknowledgments

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21. For assay protocols see Ref. 10. IC_{50} values represent the mean of at least two determinations.