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Synthesis and Structure−Activity Relationships of Benzothienothiazepinone Inhibitors of Protein Kinase D

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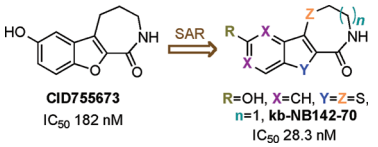
ABSTRACT Protein kinase D (PKD) is a member of a novel family of serine/threonine kinases that regulate fundamental cellular processes. PKD is implicated in the pathogenesis of several diseases, including cancer. Progress in understanding the biological functions and therapeutic potential of PKD has been hampered by the lack of specific inhibitors. The benzoxoloazepinolone CID755673 was recently identified as the first potent and selective PKD inhibitor. The study of structure−activity relationships (SAR) of this lead compound led to further improvements in PKD1 potency. We describe herein the synthesis and biological evaluation of novel benzothienothiazepinone analogues. We achieved a 10-fold increase in the *in vitro* PKD1 inhibitory potency for the second generation lead kb-NB142-70 and accomplished a transition to an almost equally potent novel pyrimidine scaffold, while maintaining excellent target selectivity. These promising results will guide the design of pharmacological tools to dissect PKD function and pave the way for the development of potential anticancer agents.

KEYWORDS Protein kinase D, small molecule inhibitor, benzothienothiazepinone, pyrimidines, CID755673



Diacylglycerol (DAG) is a key lipid secondary messenger that primarily targets protein kinase C (PKC) in cells.[1] In addition to PKC, an increasing number of DAG receptors have been discovered: protein kinase D (PKD), the chimaerin Rac GTPase-activating proteins, the Ras guanyl nucleotide-releasing proteins (RasGRPs), Munc13s, and the DAG kinases (DGKs).[2] All of these receptors are thought to be partially responsible for the diverse biological actions of DAG signaling. The PKD family has attracted particular attention because it is not only a target of DAG but also a direct substrate of PKC,[3] thus positioning itself downstream of DAG and PKC at a central position in the signal transduction pathway.[4−6]

PKDs are serine/threonine kinases that are now classified as a subfamily of the Ca^{2+}/calmodulin-dependent kinase (CaMK) superfamily.[7] To date, three PKD isoforms have been identified: PKD1 (formerly PKCμ),[8,9] PKD2,[10] and PKD3 (formerly PKCν).[11] They share a highly homologous sequence that comprises a catalytic domain and a regulatory domain. The regulatory domain of each PKD isoform is mainly constituted by a C1 domain, which binds DAG/phorbol esters, and a pleckstrin homology (PH) domain, which mediates protein−protein interactions and PKD autoinhibition.[8−11]

DAG-responsive PKC is known to activate PKD by direct interaction with the PH domain of PKD and transphosphorylation of its activation loop at the conserved Ser^{744} and Ser^{748}.[3,12] Subsequent autophosphorylation occurs at multiple sites, including Ser^{916}.[13] Furthermore, DAG regulates the intracellular localization of PKD by binding to its C1 domain.[4−6] Indeed, activated PKD seems to be mobile and able to shuttle between different subcellular compartments. Accordingly, the DAG-PKC-PKD signaling cascade represents a key mechanism that controls PKD function in cells. Recent evidence also indicates that PKD can be activated independently of PKC, adding to the complexity of the signaling network.[5] PKD contributes to a range of cellular responses, including cell proliferation, cell survival through oxidative stress-induced activation of nuclear factor-kappaB (NF-κB) signaling, gene expression by regulation of class IIa histone deacetylases, protein trafficking from the Golgi to the plasma membrane, cell motility, and immune responses.[4−6,14,15] This ubiquitous role of PKD at the cellular level accounts for its implication in pathological processes such as cardiac hypertrophy,[16,17] angiogenesis,[18,19] as well as tumor cell proliferation and metastasis,[20−23] therefore constituting a potential therapeutic target for cardiovascular diseases and oncology.[14,15]

The lack of specificity of early PKD inhibitors, i.e. the isoquinoline sulfonamide H89, staurosporine analogues, and

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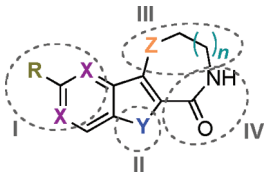
Table 1. Chemical Structure, SAR Analysis, and PKD Inhibitory Activity of CID755673 and Selected Synthetic Analogues



		structure					IC_{50}	
zone	compd	X	Y	Z	R	*n*	PKD1 (nM)[a]	cellular PKD1 (μM)[b]
	CID755673	CH	O	CH_2	OH	1	182 ± 27 (*n* = 5)	11.8 ± 4.0 (*n* = 3)
II	kb-NB123-57	CH	NH	CH_2	OH	1	130 ± 14 (*n* = 3)	> 50 (*n* = 3)
II, III	kb-NB142-70	CH	S	S	OH	1	28.3 ± 2.3 (*n* = 3)	2.2 ± 0.6 (*n* = 3)
I	kb-NB165-09	CH	S	S	OMe	1	82.5 ± 4.6 (*n* = 4)	3.1 ± 0.5 (*n* = 3)
I	mcf292-08	CH	S	S	N_3	1	74.9 ± 14.8 (*n* = 5)	2.2 ± 0.2 (*n* = 3)
III	kb-NB165-92	CH	S	S	OH	2	111 ± 6 (*n* = 3)	2.6 ± 0.7 (*n* = 2)
III	kb-NB184-02	CH	S	S	OMe	2	193 ± 27 (*n* = 3)	18.6 ± 2.0 (*n* = 3)
I	kmg-NB4-23	N	S	S	OMe	1	124 ± 31 (*n* = 4)	6.8 ± 1.3 (*n* = 3)

[a] PKD1 IC_{50} was determined using a radiometric kinase activity assay as previously described.[24] Each IC_{50} was calculated as the mean ± SEM of at least three independent experiments with triplicate determinations at each concentration in each experiment; *n* = number of independent experiments. [b] Cellular IC_{50} was determined by densitometry analysis of Western blotting data for PKD1 autophosphorylation at S^{916} in LNCaP cells as previously described.[25] Each IC_{50} was calculated as the mean ± SEM of at least two independent experiments; *n* = number of independent experiments.

resveratrol, has significantly impeded the further analysis of the regulation and biology of this kinase, but more recently, several more selective inhibitors have been reported.[14,15,17,23] A significant accomplishment in this area was recently achieved by the identification and characterization of CID755673, the first potent and selective PKD inhibitor (Table 1).[24] CID755673 inhibits PKDs with an *in vitro* IC_{50} of 182 nM for PKD1, 280 nM for PKD2, and 227 nM for PKD3. In addition to its potency, CID755673 was unique because it was not an ATP-competitive inhibitor, implying that it may bind to an alternative site on PKD and, consequently, offer a greater selectivity for PKD vs other protein kinases. CID755673 effectively blocked PKD-mediated cellular functions and revealed novel tumor-promoting functions of PKD isoforms in prostate cancer cells.[24]

Herein, we report our structure−activity relationship (SAR) efforts based on CID755673. The search for further information of the binding interaction and the identification of more potent analogues was guided by the dissection of the parent compound CID755673 into four major structural zones (Table 1): zone I (aryl moiety), zone II (furan), zone III (azepine), and zone IV (amide function).[25] The compounds resulting from the structural modifications of each of these zones were evaluated *in vitro* and in cells for their PKD1 inhibitory activity. Among the *ca.* 50 analogues that were prepared, data for the most representative in each series are summarized in Table 1.

All modifications to zone IV, which included functional group interconversions and replacement of the amide moiety, failed to enhance the inhibitory activity (data not shown).

In zone II, replacement of the oxygen in the parent compound CID755673 with a nitrogen atom resulted in equivalent or slightly enhanced potency, as exemplified by pyrrole kb-NB123-57, which showed an IC_{50} of 130 nM. Interestingly, replacement of the oxygen with a sulfur atom significantly improved the activity and led to the discovery of the potent benzothienothiazepinone kb-NB142-70, exhibiting an *in vitro* IC_{50} of 28.3 nM.

Zone I modifications included functionalization across the aryl moiety and substitutions on the phenolic hydroxyl group. Most of these zone I derivatives were less active than CID755673. Specifically, carbon substituents *ortho* to the phenol and *O*-benzylations were detrimental, whereas *ortho*-halogenation[25] had a minor effect on the *in vitro* activity (data not shown). Furthermore, phenol *O*-methylation was well tolerated, as illustrated by an IC_{50} of 82.5 nM for the methoxy analogue, kb-NB165-09. This remarkable potency of the methyl ether compared to the phenol kb-NB142-70 may indicate that a hydrogen bond donor at this position is not crucial for activity. In contrast, the dramatic loss in activity of the benzyl analogue suggests that the zone I pocket in the protein is sterically limiting. Notably, the azide analogue mcf292-08 maintained a high inhibitory activity with an IC_{50} of 74.9 nM. This result confirms the limited significance of a hydrogen bond donor at this position and also suggests that an azide group can fit quite well inside the sterically restricted binding pocket.

In zone III, the ring size was altered by addition or removal of methylene groups and substitution at the benzylic position. Concomitant to zone II modification by a thiophene, thioether insertion *exo* to the five-membered heterocycle was not detrimental to the activity, as shown for kb-NB142-70 and kb-NB165-09, although no conclusion could be drawn at this point regarding the relative contribution of each modification. Moreover, increasing the ring size from 7 to 8 atoms by inserting an additional methylene group in zone III was also well tolerated, as suggested by the potency of the

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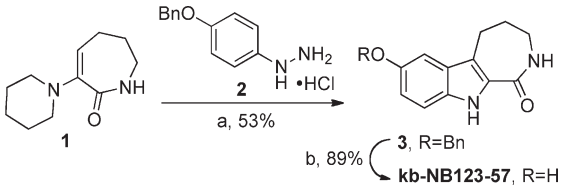
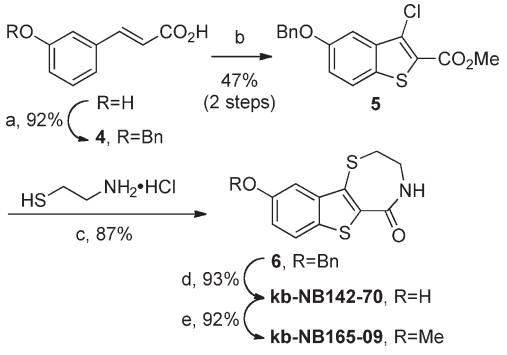
Scheme 1. Synthesis of the β-Carboline Analogue kb-NB123-57[a]



[a] Reagents and conditions: (a) H_2SO_4, EtOH, reflux, 5 h. (b) Pd/C, NH_4HCO_3, MeOH, reflux, 2 h.

Scheme 2. Synthesis of the Benzothienothiazepinone Analogues kb-NB142-70 and kb-NB165-09[a]



[a] Reagents and conditions: (a) BnBr, NaOH, EtOH, rt, 14 h. (b) (i) $SOCl_2$, pyr, DMF, PhCl, 120 °C, 22 h; (ii) Et_3N, MeOH, reflux, 12 h. (c) DBU, DMF, rt, 1.5 h; then 70 °C, 12 h. (d) BBr_3, DCM, −20 to 0 °C, 2.5 h. (e) MeI, K_2CO_3, DMF, rt, 12 h.

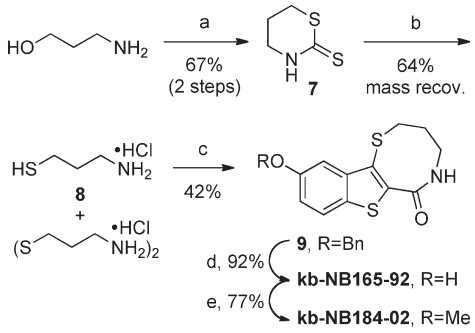
benzothienothiazocinones kb-NB184-02 (IC_{50} 193 nM) and kb-NB165-92 (IC_{50} 111 nM). It is also worth noting that in the case of the 8-membered ring, the same trend applies regarding the slightly decreased activity of the methoxy vs the free phenol substituent in zone I.

The synthesis of representative analogues in each series required the development of flexible synthetic routes. β-Carboline kb-NB123-57 was prepared from phenylhydrazine **2** *via* a Fischer-like indole synthesis with the corresponding 7-membered α-ketolactam, obtained *in situ* by acid-catalyzed hydrolysis of the enamine **1** (Scheme 1).[26] Debenzylation by transfer hydrogenation led to the phenol kb-NB123-57.[27]

Benzothienothiazepinones kb-NB142-70 and kb-NB165-09 were synthesized according to a literature protocol[28,29] beginning with benzyl protection of commercially available 3-hydroxycinnamic acid (Scheme 2). Thionyl chloride-mediated Higa cyclization of acid **4** provided the benzo[*b*]thiophene acid chloride, which was subsequently converted to methyl ester **5**. Treatment of **5** with cysteamine hydrochloride in the presence of DBU resulted in formation of the desired benzothienothiazepinone core.[29] Deprotection of the aryl benzyl ether with boron tribromide provided kb-NB142-70 in good yield, and *O*-methylation of kb-NB142-70 with methyliodide led to kb-NB165-09.

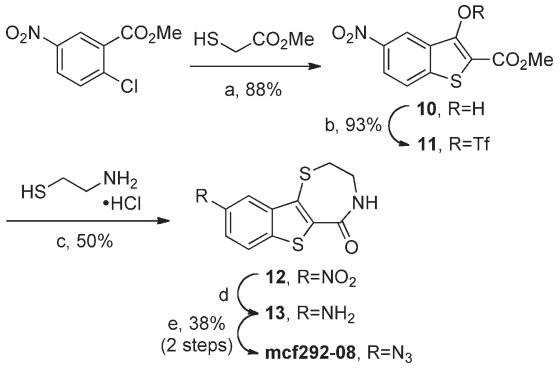
In order to synthesize the thiazocinone annulated benzothiophenes kb-NB165-92 and kb-NB184-02, the aminopropanethiol hydrochloride **8** was obtained through ring-opening

Scheme 3. Synthesis of the Benzothienothiazocinones kb-NB165-92 and kb-NB184-02[a]



[a] Reagents and conditions: (a) (i) $ClSO_3H$, CCl_4, rt, overnight; (ii) CS_2, NaOH, EtOH/H_2O, 0 °C to reflux, 40 min. (b) conc HCl, reflux, N_2, 14 days. (c) **5**, DBU, DMF, rt, 2 h; then 70 °C, 18 h. (d) BBr_3, DCM, −20 °C to rt, 2 h. (e) MeI, K_2CO_3, DMF, rt, overnight.

Scheme 4. Synthesis of the Azidobenzothienothiazepinone Analogue mcf292-08[a]



[a] Reagents and conditions: (a) Et_3N, MeOH, 40−50 °C, 4 h. (b) Tf_2O, Et_3N, DMAP, DCM, rt, 2 h. (c) DBU, DMF, rt, 1.5 h; then 70 °C, 13 h. (d) $SnCl_2$, EtOH, reflux, 5 h. (e) *t*-BuONO, $TMSN_3$, MeCN, rt, 1.5 h.

of thiazinanethione **7**, prepared in two steps from commercially available 3-amino-1-propanol (Scheme 3).[30] Aminothiol **8** was isolated as an approximately 1:1.3 thiol/disulfide mixture and used directly in the cyclocondensation−deprotection sequence to yield the desired benzothienothiazocinones.

Replacement of the phenol group by an azide on the benzothiophene scaffold required the development of an alternative route (Scheme 4). Aromatic nucleophilic substitution of methyl 2-chloro-5-nitrobenzoate by the methyl thioglycolate anion followed by immediate Dieckman cyclization[31,32] afforded the benzothiophene precursor **10**. Cyclization of the corresponding triflate **11** with cysteamine hydrochloride provided the desired tricyclic core **12** in 50% yield (67% based on recovered starting material **10**). After reduction of the nitro group, treatment of aniline **13** with *tert*-butyl nitrite and TMS-azide using Moses' method[33] yielded the aryl azide mcf292-08.

Our SAR had established that, in general, benzothienothiazepinones were superior for PKD inhibition compared to benzofuroazepinones. The benzothienothiazepinone kb-NB142-70 was the most potent analogue, with an *in vitro* IC_{50}

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of 28 nM for PKD1, achieving a nearly 7-fold improvement in potency over the earlier lead structure CID755673. This trend was further confirmed in cell-based assays, for which the cellular IC_{50} was lowered 5-fold from 11.8 μM for the inhibition of PMA-induced activation of endogenous PKD1 by CID755673 to single digit 2.2 μM by kb-NB142-70. While this result boded well for the ability of kb-NB142-70 to inhibit PKD1 in intact cells, *in vivo* studies revealed a short plasma half-life for the benzothienothiazepinone.[34] Accordingly, we further explored a zone I modification of kb-NB142-70 to install a more electron-deficient pyrimidine moiety in place of the phenol ether, a known site of active phase I and II metabolism.

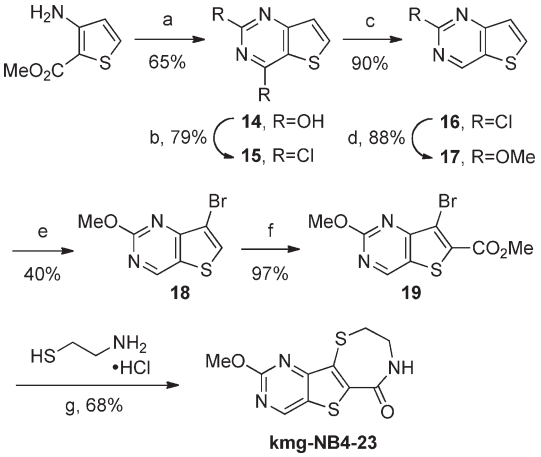
The synthetic route to arrive at this new thiazepinothiophenopyrimidinone scaffold is summarized in Scheme 5. Starting with commercially available methyl 3-aminothiophene-2-carboxylate, formation of the pyrimidine moiety using potassium cyanate, followed by chlorination with $POCl_3$ gave dichloride **15**.[35] Palladium-catalyzed regioselective dechlorination of **15** in the presence of Na_2CO_3 occurred exclusively at the C-4 position,[36] and substitution of the remaining C-2 chloride with methoxide provided **17** in 79% yield over the two steps. Electrophilic bromination of **17** using bromine in AcOH[37] gave the desired C-7 brominated compound **18**. Functionalization at C-6 under Knochel's conditions[38] gave the required cyclization precursor **19**. Formation of the thiazepinone moiety was then achieved by one-pot nucleophilic displacement−condensation of **19** with cysteamine hydrochloride, providing the desired methoxypyrimidine kmg-NB4-23 in good yield.

Gratifyingly, pyrimidine kmg-NB4-23 exhibited an IC_{50} of 124 nM, which is only a slight decrease in activity compared to that of the parent lead kb-NB165-09. This encouraging result thus confirms the validity of our design and also suggests that decreased π-electron density is well tolerated in the aryl region.

In order to establish the selectivity profile of our analogue series against other kinases, we also performed a variety of counterscreens using AKT, CAK (CDK7), PLK1, PLK2, several PKCs, and CaMKα. In contrast to the low nanomolar activity of our analogues against PKD, none of our inhibitors were found to be significantly active at concentrations < 10 μM in these screens. Furthermore, we tested the selectivity of our leads kmg-NB4-23, mcf292-08, and kb-NB123-57 against PKCα, PKCβI, and PKCδ, and we did not detect significant activities in these assays. For CaMKIIα, we found weak ($< 40\%$) inhibition at 10 μM for mcf292-08 and kb-NB123-57. These counterscreens clearly establish a high selectivity profile for PKD inhibition in our analogue series.[39]

We have also started to probe the cellular effects of kmg-NB4-23, mcf292-08, and kb-NB123-57.[39] The azidobenzothienothiazepinone mcf292-08 inhibited PMA-induced PKD1 S^{916} autophosphorylation in LNCaP prostate cancer cells with an IC_{50} of 2.2 μM. For the thiazepinothiophenopyrimidinone kmg-NB4-23, an IC_{50} of 6.8 μM was found in this assay. In contrast, kb-NB123-57 did not significantly inhibit the PMA-induced PKD1 autophosphorylation at S^{916} ($IC_{50} > 50$ μM), possibly due to limited membrane permeability, adverse protein binding, metabolic degradation, or other compensatory pathways in cells.

Scheme 5. Synthesis of the Thiazepinothiophenopyrimidinone Analogue kmg-NB4-23[a]



[a] Reagents and conditions: (a) (i) KOCN, AcOH, H_2O, rt, 20 h; (ii) 2 N NaOH, rt, 2 h. (b) $POCl_3$, MeCN, reflux, 2 days. (c) H_2, Pd/C, EtOH, Na_2CO_3, rt, 24 h. (d) NaOMe, MeOH, reflux, 6 h. (e) AcOH, Br_2, 70 °C, 24 h. (f) (i) TMPMgCl·LiCl, −50 °C, 2 h; (ii) $MeCO_2CN$. (g) DBU, DMF, rt, 1.5 h; then 70 °C, 10 h.

In summary, we have identified a series of novel benzothienothiazepinones as PKD inhibitors. A first round of optimization from the initial lead CID755673 led to the discovery of kb-NB142-70 and its methoxy analogue kb-NB165-09. These new lead compounds inhibited PKD1 *in vitro* in the low nanomolar range, achieving a nearly one-log IC_{50} enhancement compared to the HTS hit CID755673. Additionally, these inhibited PKD1 autophosphorylation at Ser^{916} in LNCaP prostate cancer cells in the low micromolar range, a 10-fold improvement compared to the case of CID755673. In order to circumvent metabolism issues, further core modification with a pyrimidine moiety led to the promising new lead structure kmg-NB4-23. SAR studies are currently in progress to improve the activity of kmg-NB4-23 as well as its selectivity toward PKD1 vs PKD2 or PKD3, for which the IC_{50} values were found in the 50−500 nM range for all derivatives tested (data not shown). In conclusion, our results to date demonstrate that modifications of CID755673 significantly modulate its potency but have only limited effect on isoform selectivity.[25] This suggests that the binding domain may be highly conserved among the three isoforms. Finally, the azide analogue mcf292-08, also active at nanomolar (*in vitro*) or micromolar (in cells) concentrations, represents a valuable tool for future photoaffinity labeling studies that may provide key structural information for binding site identification.

SUPPORTING INFORMATION AVAILABLE

Experimental procedures, assay results, and spectral data for compounds. This information is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions: Karla Bravo-Altamirano participated in research design (chemistry) and execution (performed synthesis); Marie-Céline Frantz participated in research design (chemistry) and execution (performed synthesis) and contributed to writing of the manuscript; Kara M. George participated in research design (chemistry) and execution (performed synthesis) and contributed to writing of the manuscript; Courtney R. LaValle participated in research execution (performed screens); John S. Lazo participated in research design (biology), acquired funding, and contributed to writing of the manuscript; Stephanie Leimgruber participated in research execution (performed counterscreens); Elizabeth R. Sharlow participated in research design (biology) and execution (performed counterscreens); Manuj Tandon participated in research execution (performed screens); Q. Jane Wang led research design (biology), acquired funding, and contributed to writing of the manuscript; and Peter Wipf led research design (chemistry), acquired funding, contributed to writing of the manuscript, and is the corresponding author.

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ABBREVIATIONS CAK, CDK activating kinase; CaMK, Ca^{2+}/calmodulin-dependent kinase; CDK, cyclin-dependent kinase; DAG, diacylglycerol; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DGK, DAG kinase; DMAP, *N*,*N*-dimethylaminopyridine; HDAC, histone deacetylase; NF-κB, nuclear factor-kappaB; PH, pleckstrin homology; PKC, protein kinase C; PKD, protein kinase D; PLK, polo-like kinase; PMA, phorbol 12-myristate 13-acetate; RasGRP, Ras guanyl-nucleotide-releasing protein; SAR, structure—activity relationship; TMP, 2,2,6,6-tetramethylpiperidine.

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