Design, synthesis and biological evaluation of pyrazolo[3,4-*d*]pyrimidine-based protein kinase D inhibitors

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Highlights

- A novel series of pyrazolo[3,4-*d*]pyrimidine based PKD inhibitors with structural variety at position 1 was synthesized.
- **17m** showed the most potent pan-PKD inhibitory activity with IC₅₀ values ranging from 17 to 35 nM.
- **17m** and 3-IN-PP1 potently blocked PKD-dependent cortactin phosphorylation in PANC-1 cells.
- 3-IN-PP1 exhibited low micromolar cytotoxic activity against various cancer cell lines.

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ABSTRACT

The multiple roles of protein kinase D (PKD) in various cancer hallmarks have been repeatedly reported. Therefore, the search for novel PKD inhibitors and their evaluation as antitumor agents has gained considerable attention. In this work, novel pyrazolo[3,4-*d*]pyrimidine based pan-PKD inhibitors with structural variety at position 1 were synthesized and evaluated for biological activity. Starting from 3-IN-PP1, a known PKD inhibitor with IC₅₀ values in the range of 94-108 nM, compound **17m** was identified with an improved biochemical inhibitory activity against PKD (IC₅₀ = 17-35 nM). Subsequent cellular assays demonstrated that 3-IN-PP1 and **17m** inhibited PKD-dependent cortactin phosphorylation. Furthermore, 3-IN-PP1 displayed potent anti-proliferative activity against PANC-1 cells. Finally, a screening against different cancer cell lines demonstrated that 3-IN-PP1 is a potent and versatile antitumoral agent.

1. Introduction

Protein kinase D (PKD) is a serine/threonine kinase that belongs to the Ca²⁺/calmodulin-dependent kinase group and comprises three isoforms that share high sequence identity: PKD1, PKD2 and PKD3.[1] These isoforms all contain a conserved catalytic domain, a regulatory pleckstrin homology (PH) domain and two N-terminal cysteine-rich, zinc finger-like motifs. Additionally, PKD 1 and 2 have a PDZ-binding motif at the very end of their C-terminus.[2, 3]

The PKD family is repeatedly associated with multiple cancers such as prostate, breast, colorectal, pancreatic, gastric cancer and glioblastoma.[4] Indeed, PKD activity is related to a plethora of physiological conditions and dysregulated PKD function is linked to multiple oncogenic processes including cell growth and proliferation, cell survival, motility, apoptosis inhibition, angiogenesis and epidermal to mesenchymal transition (EMT).[5, 6] The existence of PKD isozymes introduces an additional layer of complexity in the cell signaling of these malignancies, as these isoforms can either promote or reduce tumor formation.[3] PKD1

has tumor-suppressive roles in prostate, breast and colorectal cancer and is able to reduce EMT via the phosphorylation of Snail.[7-9] Furthermore, PKD1 expression is significantly downregulated in invasive breast cancer cells due to epigenetic hypermethylation of the *PRKD1* gene promoter, hence further strengthening its potential role in repressing tumor growth.[10] In addition, global knockout (KO) of PKD1 in mice results in embryonic lethality, while KO PKD2 mice are viable, thereby signifying PKD1's vital role.[11, 12] On the other hand, PKD2 is markedly more linked to processes that mediate cancer growth. For example, it is a crucial regulator of angiogenesis through endothelial cell proliferation and migration.[13] Furthermore, depletion of PKD2 by inhibition of HSP90 is associated with increased cell death of several human cancer cell lines.[14] Recent studies show that PKD2 is the major isoform in human colon cancer cells and PKD2 silencing studies reveal significant downregulation of AKT and ERK, kinases that are both involved in cell proliferation.[15] In addition, downregulation of PKD2 prevents glioblastoma tumor growth, presumably via suppression of cyclin D1.[16]

Figure 1. Reported PKD inhibitors

Remarkably, PKD2 silencing in pancreatic cancer cells orthotopically implanted in mice significantly impairs tumor angiogenesis and growth, thus indicating its crucial role.[17] In comparison with PKD1 and PKD2, PKD3 is studied to a much lesser extent. Nevertheless, it has been categorized as tumor promoting.[18, 19] Overall, it is becoming more and more clear that the PKD family members have isozyme-specific roles within the cell. However, a complete understanding of their exact roles in both physiological and pathological conditions remains to be uncovered.

Despite the opposing roles of the PKD isozymes in cancer, pan-PKD inhibitors do show antitumoral activity in various cancer models, both *in vitro* and *in vivo* (Figure 1). Benzoxoloazepinolone CID755673 and its derivatives are potent, ATP non-competitive, PKD inhibitors that reduce cell proliferation, cell migration and invasion in several prostate cancer cell lines (such as LNCaP and PC3).[20, 21] The selectivity of this compound, however, remains unclear as PKD1-independent biological effects were observed and kinase off-targets were identified.[22, 23] The same group also discovered pteridine analogue SD-208 as a potent PKD inhibitor. Treatment of PC3 xenograft mice with SD-208 reduced tumor growth and enhanced apoptosis.[24] In addition, SD-208 also reduced prostate cancer cell invasion, which might be related to off-target inhibitory activity towards TGF- β RI. CRT0066101 is a potent pan-PKD inhibitor exhibiting remarkable *in vivo* anti-cancer potency. Treatment of both hetero- and orthotopic PANC-1 xenograft mice with CRT0066101 leads to a significant tumor growth reduction.[25] In addition, CRT0066101 shows also promising activity in various colorectal cancer models including the HCT-116 cell lines and HCT-116 mice xenografts.[15] Moreover, CRT0066101 significantly reduces tumor growth of both estrogen receptor negative as well as triple negative breast cancer.[26, 27] Although the specificity of CRT0066101 against PKD was confirmed in a panel of more than 90 kinases,[25] doubts about the selectivity were raised.[15]

Besides their anti-cancer applications, PKD inhibitors based on other chemotypes have been profiled in other disease areas. Examples include the 3,5-diarylisoxazoles and 2,6-naphthyridines that both potently inhibit PKD and have been investigated as potential treatment of cardiac hypertrophy.[28, 29]

We and others have been investigating derivatives of pyrazolo[3,4-*d*]pyrimidine 1-NA-PP1 as PKD inhibitors.[30, 31] 1-NA-PP1 was originally designed as a specific inhibitor of analog sensitive v-src protein kinases.[32, 33] These kinases were generated via site-directed mutagenesis of their gatekeeper residue, thereby creating a unique back pocket able to accommodate the naphthalene moiety. However, 1-NA-PP1 and its close derivative 1-NM-PP1 do have off-targets against a number of non-mutated protein kinases, such as wild-type PKD.[34] Elaborating on these findings, we previously discovered that replacement of the naphthalene moiety of 1-NA-PP1 with an indole substituent (3-IN-PP1) further increased the inhibitory potency against PKD by tenfold.[31] Since no X-ray crystallographic structures of the catalytic domain of PKD are available, we rationalized our findings with an in-house generated homology model of PKD. We speculated that the increased PKD inhibition was due to an alternative binding mode which has also been observed for other pyrazolo[3,4-*d*]pyrimidine based transferase inhibitors. These novel insights encouraged us to further explore the structure-activity relationship (SAR) of the pyrazolo[3,4-*d*]pyrimidines as PKD inhibitors, focusing on the largely ignored 1-position. Furthermore, in the course of this work, off-target PKD activity was observed in a chemoproteomic profiling study of BKI1369, a pyrazolopyrimidine derived bumped kinase inhibitor (BKI) designed to target parasitic kinase *Toxoplasma gondii* calcium-dependent protein kinase 1 (TgCDPK1).[35] This pyrazolo[3,4-*d*]pyrimidine contains an ethoxyquinolyl substituent in the 3-position and a methylene piperidine substituent in the 1-position. In the present study, we report the design and synthesis of novel 3-IN-PP1 analogues and their biochemical evaluation as PKD inhibitors. The most promising PKD inhibitors were evaluated for *in cellulo* inhibition of PKD and their effect on cell proliferation in a pancreatic adenocarcinoma cell line (PANC-1). Finally, a selection of compounds was screened for their cytotoxic activity in a panel of eight different cancer cell lines.

2. Results and Discussion

2.1 Chemistry

The pyrazolo[3,4-*d*]pyrimidines were synthesized according to protocols reported by Todorovic and Bishop.[32, 36] Conventional methods to functionalize the 3-position of this pyrazolopyrimidine scaffold rely on either palladium-catalyzed cross-coupling reactions or introduction of the side chain prior to the heterocycle construction, if sp²-sp³ bond formation is required. The synthesis of methylene bridged 3-IN-PP1 is shown in Scheme 1. *N*-tosyl-protected 2-(indol-3-yl)acetic acid **4** was prepared from commercially available indole-3-acetic acid **1**. Subsequent condensation with malononitrile, followed by *O*-methylation with dimethyl sulfate afforded compound **6**. A first cyclization of **6** with *tert*-butyl hydrazine allowed to construct the pyrazole moiety yielding intermediate **7** and a second ring closure with formamide formed the pyrimidine ring furnishing protected pyrazolo[3,4-*d*]pyrimidine **8**. Finally, deprotection of the indole moiety under basic conditions yielded methylene-linked 3-IN-PP1 derivative **9**.

As part of the SAR study at the 3-position, the synthesis of functionalized indolyl derivatives was envisioned. Previously, 3-IN-PP1 was synthesized via a Suzuki-coupling between 3-bromo-1-(*tert*-butyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine and 1-(phenylsulfonyl)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-indole.[31] The latter was prepared from indole in three steps including protection of the indole nitrogen atom and selective bromination at position 3, followed by borylation. After the cross-coupling reaction, a final deprotection step was performed. This laborious route prompted us to explore alternative synthetic procedures. Inspired by the Smith group's work on selective borylation, 3-borylated 1*H*-indole **11a** was prepared in one step from indole using pinacolborane (HBpin) and a phenantroline/iridium catalytic system.[37] Subsequently, this unprotected borylated indole **11a** was successfully coupled to 3-bromo-pyrazolo[3,4-*d*]pyrimidin-4-amine **10** (synthesized from (ethoxymethylene)malononitrile according to a literature procedure), yielding compound **12a** (3-IN-PP1). Using a similar methodology, a set of functionalized indoles, a pyrrole and a pyrazole were borylated furnishing intermediates **11b-11h** (Scheme 2). Subsequent coupling with 3-bromo-pyrazolo[3,4-*d*]pyrimidine **10** yielded compounds **12b-h**.

Scheme 1. Synthesis of the methylene bridged 3-IN-PP1 derivative **9**. Reagents and conditions: (a) MeOH, PTSA, reflux, overnight; (b) TSCl, Et₃N, DCM, rt; (c) LiOH, MeOH, rt, overnight; (d) i. SOCl₂, reflux; ii. malononitril, NaH, THF, 0 °C; (e) Me₂SO₄, NaHCO₃, dioxane/H₂O; (f) *tert*-butyl hydrazine hydrochloride, Et₃N, EtOH, 2 h; (g) formamide, reflux; (h) NaOH, EtOH, reflux, 1 h.

Scheme 2. Synthesis of 3-functionalized 3-IN-PP1 derivatives. Reagents and conditions: (a) i. HBpin, Et₃N, THF, 80 °C, 20 min; ii. [Ir(OMe)(COD)]₂, 3,4,7,8-tetramethyl-1,10-phenanthroline, 80 °C, 4 h; [Ir(OMe)(COD)]₂, 3,4,7,8-tetramethyl-1,10-phenanthroline, 80 °C, 4 h; (c) Boronic acid pinacol ester, K₂CO₃, Pd(PPh₃)₄, dioxane/water (4/1), 150 °C, Microwave (MW), 15 min.; (d) MeI, KOH, DMF, rt.; (e) Pd(dba)₂, PCy₃, B₂Pin₂, KOAc, dioxane, 80 °C, overnight.

Scheme 3. Synthesis of 1-functionalized pyrazolo[3,4-*d*]pyrimidines. Reagents and conditions: (a) Alkyl mesylate or halide, Cs₂CO₃, DMF, 80 °C, 4 h; (b) Alcohol, DIAD, PPh₃, THF, 0 °C, 1 h; (c) Boronic acid pinacol ester, K₂CO₃, Pd(PPh₃)₄, dioxane/water (4/1), 150 °C, MW, 15 min.; (d) Pd/C, THF, 50 °C, 8 h.

Quinoline derivative **11i** was synthesized using a palladium-catalyzed borylation of 5-bromoquinoline and was subsequently coupled similarly to 3-bromo-pyrazolo[3,4-*d*]pyrimidine **10** to afford **12i**. Finally, using iodomethane under alkaline conditions, the nitrogen of the indolyl moiety of 3-IN-PP1 (**12a**) was methylated affording compound **13**.

For the synthesis of pyrazolo[3,4-*d*]pyrimidine analogues with structural variation at position 1, we were inspired by the work of Todorovic.[36] Iodination of 1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine using NIS is followed by alkylation and cross-coupling. The alkylation is carried out either *via* S_N2 reactions, using an alkyl mesylate or alkyl halide, or a Mitsunobu reaction. A diverse set of hydrophobic and hydrophilic substituents was introduced at the 1-position (**16a-l**, **16n**) followed by a Suzuki coupling with 3-boryl-1*H*-indole affording the desired 3-IN-PP1 analogues **17a-l** and **17n** (Scheme 3). In order to have a methylene piperidinyl substituent at position 1, -Boc was initially selected as protecting group for the nitrogen atom of the piperidine moiety. Unfortunately, multiple efforts to cleave the Boc-group under acidic conditions failed to yield pure **17m**. Therefore, we opted for a -Cbz protecting group and its reductive deprotection yielded the corresponding free amine **17m** in excellent yield. In addition, two analogues containing a naphthyl substituent instead of the indolyl moiety (**18m** and **18n**) were prepared. BKI1369 was synthesized following a slightly modified version of the original procedure reported by Vidadala *et al.* (see Supporting Information).[38]

2.2 PKD inhibition

All compounds were evaluated for PKD inhibition against full-length PKD using Promega's ADP-Glo™ kinase activity assay and syntide-2 as a substrate. The known PKD inhibitors 1-NM-PP1 and 3-IN-PP1 were included as reference compounds. In a first round of screening, all compounds were evaluated at a single concentration of 1 μM and the data were expressed as the percentage of residual PKD2 activity. The first series encompassed a focused library with structural variation at position 3 of the pyrazolo[3,4-*d*]pyrimidine scaffold (Table 1). Previously, we demonstrated that 1-NM-PP1 was a more potent PKD2 inhibitor compared to 1-NA-PP1. Introducing a similar methylene bridge in 3-IN-PP1 (**12a**) yielded compound **9** that unfortunately was less potent than 3-IN-PP1 against PKD2 (48% versus 15% residual activity). The methylated 3-IN-PP1 analogue **13** showed significantly reduced PKD2 inhibition (53% residual activity), when compared to 3-IN-PP1 (15% residual activity), indicating the involvement of this nitrogen atom in hydrogen bonding. The introduction of subtle decorations on the indole moiety (e.g. chlorine, fluorine and methoxy substituents) afforded compounds **12b-12e** that were completely devoid of PKD2 inhibition (72-97% residual activity). In addition, pyrrole **12f**, pyrazole **12g** and quinoline **12i** lacked activity against PKD2. Furthermore, replacing the indole substituent of 3-IN-PP1 for an azaindole group resulted in a compound (**12h**) that is only slightly less active than 3-IN-PP1. Together, these observations further demonstrate the very narrow space for improvements at the 3-position which is in complete agreement with Wipf's findings and our previous work.[30, 31]

In contrast, the 1-position of the pyrazolo[3,4-*d*]pyrimidine scaffold is significantly understudied since only methyl and *tert*-butyl substituents have been extensively evaluated. A library of

pyrazolo[3,4-*d*]pyrimidines with bulkier substituents at position 1 was evaluated for PKD2 inhibition (Table 2). The introduction of a single methylene linker between the pyrazolo[3,4-*d*]pyrimidine scaffold and the *tert*-butyl substituent gave the neopentyl derivative **17a** that was slightly less potent than 3-IN-PP1 (25% versus 15% residual PKD2 activity). The insertion of an ethylene linker (affording neoheptyl analogue **17b**) led to a complete loss of PKD2 inhibition. The presence of larger cycloaliphatic substituents (**17c-e**) or aromatic moieties (**17f-i**) afforded compounds with strongly reduced PKD2 inhibitory potency (between 43-100% residual PKD2 activity) in comparison to 3-IN-PP1. As the incorporation of these bulky hydrophobic substituents was detrimental for PKD inhibition, the influence of polar groups was assessed. Notably, the pyranyl analogue **17j** and the *N*-acetylpiperidinyl derivative **17k** are more potent PKD2 inhibitors when compared to the corresponding cyclohexyl analogue **17d**, indicating that more polar substituents are better accommodated. Based on the potent off-target PKD inhibition of BKI1369 (*vide supra*), a methyl piperidinyl and a 2,2-dimethylbutan-1-ol group at position 1 were both evaluated, while keeping the indole moiety at position 3. Remarkably, both **17m** and **17n** displayed excellent PKD2 inhibition with almost no remaining kinase activity when tested at a concentration of 1 μM. The naphthyl derivatives **18m** and **18n** are both less potent PKD2 inhibitors when compared to their indole-substituted congeners **17m** and **17n**, respectively. This is in agreement with what previously has been observed for 1-NA-PP1 and 3-IN-PP1.[31]

Table 1. PKD2 inhibition of 3-substituted pyrazolo[3,4-*d*]pyrimidines

CPD	R ¹	RA (%) ^a	CPD	R ¹	RA (%) ^a
1-NM-PP1		20 ± 1	12d		72 ± 5
3-IN-PP1		15 ± 1	12e		97 ± 7
9		48 ± 2	12f		68 ± 26
13		53 ± 10	12g		94 ± 9
12b		96 ± 7	12h		25 ± 3
12c		82 ± 2	12i		82 ± 1

^a Determined using ADP-Glo™ kinase assay; CPD = Compound; RA = residual activity at 1 μM.

Table 2. PKD2 inhibition of 1-substituted pyrazolo[3,4-*d*]pyrimidines

CPD	R ¹	R ²	RA (%) ^a	CPD	R ¹	R ²	RA (%) ^a	CPD	R ¹	R ²	RA (%) ^a
17a			25 ± 1	17f			68 ± 4	17k			44 ± 1
17b			93 ± 1	17g			69 ± 7	17m			5 ± 2
17c			43 ± 3	17h			100	18m			19 ± 3
17d			92 ± 8	17i			96 ± 4	17n			4 ± 1
17e			100	17j			46 ± 2	18n			27 ± 5

^a Determined using ADP-Glo™ kinase assay; CPD = Compound; RA = residual activity at 1 μM.

The most promising PKD2 inhibitors were subjected to dose-response curves in order to determine the half maximal inhibitory concentration (IC₅₀) against PKD1, 2 and 3 (Table 3 and Figure S1 in Supporting Information). To have a direct comparison with known PKD inhibitors, CRT0066101 and 1-NM-PP1 were included as a benchmark in this study. The observed trends between 3-IN-PP1 and these references are in agreement with literature data.[25, 31] Furthermore, the off-target PKD activity of BKI1369 relative to the current state-of-the-art was also investigated. BKI1369 is slightly less active against PKD1 in comparison to the other two isoforms. This difference was also reported in the above-mentioned chemoproteomic profiling study.[35]

Table 3. IC₅₀ values expressed in nM for a subset of the synthesized pyrazolo[3,4-*d*]pyrimidine analogues, CRT0066101, 1-NM-PP1 and BKI1369

	Compound	IC ₅₀ PKD1 (nM) ^a	IC ₅₀ PKD2 (nM) ^a	IC ₅₀ PKD3 (nM) ^a
References	CRT0066101	16 ± 2	9 ± 2	6 ± 2
	1-NM-PP1	1013 ± 200	786 ± 40	616 ± 110
	BKI1369	183 ± 24	48 ± 2	43 ± 6
	3-IN-PP1	108 ± 20	94 ± 4	108 ± 33
	17m	35 ± 10	35 ± 5	17 ± 8
	18m	64 ± 9	55 ± 9	78 ± 2
	17n	50 ± 12	43 ± 5	76 ± 4
	12h	343 ± 98	282 ± 25	456 ± 27
	13	3319 ± 260	3286 ± 270	4055 ± 680

^a Determined using ADP-Glo™ kinase assay

The replacement of the *tert*-butyl group of 3-IN-PP1 by a 4-methylpiperidine moiety afforded compound **17m**, which is 3- to 5-fold more potent against PKD than 3-IN-PP1. With IC₅₀ values of 35, 35 and 17 nM for PKD1, 2 and 3 respectively, compound **17m** is the most potent pyrazolo[3,4-*d*]pyrimidine based PKD inhibitor reported to date. Compound **17n**, bearing a 2,2-dimethylpropan-3-ol moiety, showed only slightly higher IC₅₀ values when compared to compound **17m**, which further confirms the crucial role of a hydrogen bond donor moiety in the substituent at position 1. As expected, the naphthalene analogue **18m** was less potent; fully correlating with the observed trends between 3-IN-PP1 and 1-NA-PP1.[31] As the presence of an indolyl group at position 3 was optimal for PKD inhibition, we also investigated subtle structural modifications of this moiety. Interestingly, the *N*-methylated 3-IN-PP1 analogue (**13**) is a 30- to 40-fold less potent PKD inhibitor when compared to 3-IN-PP1, whereas aza-indole analogue **12h** displays only a 3- to 4-fold drop in PKD inhibitory activity.

2.3 Molecular docking studies.

We further investigated the binding mode of this class of compounds in the ATP binding site of PKD. According to our previously developed model, an alternative binding mode was hypothesized that better correlated with 3-IN-PP1's increased activity.[31] This alternate binding mode is also observed in X-ray crystallographic studies of APH(3')-Ia, an aminoglycoside phosphotransferase of *Acinetobacter baumannii* with an active site fold similar to protein kinases (PDB ID: 4GKI, 4GKH). With our expanded activity dataset at hand, including the library of 1-functionalized 3-IN-PP1 derivatives, we further evaluated this binding mode hypothesis. Since no X-ray structures of PKD's catalytic domain are publicly available, a homology model was constructed.

Figure 2. (A) ROC Analysis of the binding mode of pyrazolo[3,4-*d*]pyrimidine based PKD inhibitors. Activity cut-off 50% inhibition. Blue: ROC based on score of the classical binding mode. Green: ROC based on the highest score retrieved. Red: ROC obtained for the alternative binding mode. AUC = area under the curve. (B) Predicted binding mode of compound **17m** after docking into the homology model of PKD (PDB ID 3MWU as template).

As discussed above, some bumped kinase inhibitors of *Tg*CDPK1 display cross-reactivity against PKD, thus suggesting that both kinases might share a similar environment in the active site. Therefore, the crystallographic X-ray structure of *Tg*CDPK1 in complex with RM-1-95, in classic binding mode, was chosen as a template (PDB ID: 3MWU).

Our dataset containing 66 compounds (Table S1 in Supporting Information) was docked into the ATP-pocket using GOLD. Among these 66 docked ligands, 33 retrieved the classical binding mode as the primarily scored pose. To assess the fitness of each binding mode to the dataset, receiver operating characteristic (ROC) curves were constructed for three scores: the highest retrieved score, the classical binding mode score and the alternative binding mode score (Figure 2A). The curve designated by the black dashed line ($X=Y$) displays random behavior (AUC = 0.50). When considering the classic binding mode an AUC of 0.83

is obtained, thus showing good fitness. On the other hand, the AUC dramatically eroded to 0.56 when evaluating the alternative binding mode (red curve). Furthermore, the highest docking score only moderately correlated with the activity data (AUC = 0.69), presumably due to docking power related limitations. As a control, eliminating the bias of the ligand on the homology model, we also created a structural model relying on the aforementioned aminoglycoside phosphotransferase APH(3')-Ia (PDB ID: 4GKI). This transferase shares a similar fold and has 1-NM-PP1 bound in the alternative, inverted binding mode. Interestingly, also the classic binding mode was preferred over the alternative in this second homology model, thus ruling out binding mode preferences imposed during the homology modelling (Figure S3 in Supporting Information). Taken together, these results indicate that the activity profiles of our dataset generally correlate better with the classical binding mode rather than the earlier hypothesized alternative mode. Despite this observed preference, it should be noted that binding modes can be highly compound dependent and only co-crystals can reveal more case-specific details. Figure 2B displays the postulated binding mode for compound **17m**, which is similar to the one observed for inhibitor RM-1-95 in the crystal structure of *Tg*CDPK1.[39] The increased potency of **17m** can be explained by two hydrogen bonds formed by the indole and piperidine nitrogen atoms. Similar interactions are observed for the nearly equally active compound **17n**, also containing this dual hydrogen bond pattern. In our SAR study, both of these interactions were found to be crucial for potent PKD inhibition. The methylated analogue **13** was significantly less active and the importance of the hydrogen bond donor on the substituent at position 1 was also demonstrated. Furthermore, the orientation of the indole moiety resembles that of a recently reported pyrazolo[3,4-*d*]pyrimidine based covalent EGFR kinase inhibitor (PDB ID: 5J9Z).[40]

Figure 3. The effect of 1-NM-PP1, 3-IN-PP1 and **17m** on PKD mediated phosphorylation of cortactin at S298. PANC-1 cells co-transfected with FLAG-PRKD1 or FLAG-PRKD2 and myc-CTTN were pretreated with the indicated compounds (20 μ M) for 1 hour prior to stimulation with 250 nM phorbol 12,13-dibutyrate (PDBu) for 15 min. Phosphorylation of cortactin was followed via western blot. Quantification of pS298/MyC-Cort signals of three independent experiments is shown in the graph. Graph bars and error bars represent the normalized mean and calculated standard error of the mean (SEM), respectively. A one-way ANOVA was performed to determine the statistical significance with the control conditions (DMSO). EV = empty vector.

2.4 PKD inhibition in PANC-1 cells reduces cortactin phosphorylation

Cellular PKD activity was monitored by analyzing the levels of phosphorylation at S298 of cortactin (CTTN), a known substrate of PKD.[41] Both PKD 1 and 2 were investigated for PKD-dependent cortactin phosphorylation in PANC-1 cells and CRT0066101 was used as a positive control. After stimulating the cells with phorbol 12,13-dibutyrate (PDBu), all tested inhibitors significantly decreased the levels of pS298 CTTN in comparison to the control (Figure 3). Hence, all compounds are clearly inhibiting PKD in this cellular environment. Although **17m** is a more potent PKD inhibitor than 3-IN-PP1 in the biochemical assay, it appears that it is less capable of inhibiting PKD-mediated CTTN phosphorylation in a cellular environment. When PDBu stimulated pancreatic cancer cells (PANC-1) were treated with **17m**, residual CTTN phosphorylation was noticed. This was also observed for the less potent inhibitor 1-NM-PP1. In contrast, treatment with 3-IN-PP1 resulted in minimal residual PKD activity. As expected for pan-PKD inhibitors, no differences were observed between inhibition of PKD 1 and 2. Furthermore, the biochemical inhibitory activity of 3-IN-PP1 against CAMKII α and PKC δ , two related kinases, was investigated. No notable inhibition was observed at compound concentrations of 1 and 10 μ M (Figure S2 in Supporting Information).

2.5 3-IN-PP1 reduces PANC-1 cell proliferation.

The effect of PKD inhibitors **17m**, 3-IN-PP1 and 1-NM-PP1 on the proliferation of PANC-1 cells was determined (Figure 4). 3-IN-PP1 (at a concentration of 5 μ M) significantly inhibited PANC-1 cell proliferation after incubation for 96 ($p=0.041$) and 144 hours ($p<0.001$), thus performing better than the same concentration of 1-NM-PP1. Despite its more potent PKD inhibition, **17m** does not significantly affect PANC-1 cell proliferation at a concentration of 5 μ M. This correlates very well with the data from the cellular cortactin phosphorylation study in which **17m** was also less potent. A reduced cellular permeability or increased metabolism in comparison to 3-IN-PP1 may account for these effects.

Figure 4. Cell proliferation of PANC-1 cells in presence of 1-NM-PP1, 3-IN-PP1 and **17m** at a concentration of 5 μ M. 3-IN-PP1 significantly reduces PANC-1 cell proliferation at 96 hours ($p = 0.041$) and 144 hours ($p < 0.001$). A two-way ANOVA was performed using Dunnett's multiple comparisons test.

2.6 antitumoral screening reveals 3-IN-PP1 as a broad-spectrum anticancer agent.

A selection of the newly synthesized PKD inhibitors (compounds **13**, **17n** and **17m**), as well as a number of known PKD inhibitors (CRT0066101, 1-NM-PP1, 3-IN-PP1 and BK1369) were evaluated for their antitumoral activity (Table 4) in a panel of eight different cancer cell lines: LN-229 (glioblastoma), Capan-1 (pancreatic adenocarcinoma), HCT-116 (colorectal carcinoma), NCI-H460 (lung carcinoma), DND-41 (acute lymphoblastic leukemia), HL-60 (acute myeloid leukemia), K-562 (chronic myeloid leukemia) and Z-138 (non-Hodgkin lymphoma). Inhibition of cell proliferation was monitored in real-time over a period of three days using an IncuCyte live-cell automated imager determining cellular confluence as a measure of cellular viability. Overall, CRT0066101 was the most potent antiproliferative agent with IC_{50} values in the range of 0.6-2.2 μ M against the various tumor cell lines. Among the pyrazolo[3,4-*d*]pyrimidine based PKD inhibitors, 3-IN-PP1 was the most potent cytotoxic agent with IC_{50} values in the low μ M range for most cancer cell lines, with the exception for the glioblastoma cell line for which the activity was clearly less ($IC_{50} = 39.2 \mu$ M). Overall, in most tumor cell lines (except for LN-229), 3-IN-PP1 was only slightly less active than the reference PKD inhibitor CRT0066101. On the other hand, 3-IN-PP1 is much more potent as antitumoral agent than the reference pyrazolo[3,4-*d*]pyrimidine based PKD inhibitor 1-NM-PP1. The *N*-methyl-indole analogue of 3-IN-PP1 (compound **13**) was endowed with dramatically reduced anti-proliferative properties. This was completely in line with the weak PKD inhibition for compound **13**. Despite their potent activity against PKD, compound **17n** and especially compound **17m**, displayed only weak antiproliferative activity. This was in agreement with the studies on cellular cortactin phosphorylation and PANC-1 proliferation in which compound **17m** displayed overall weaker cellular activity. In addition, pyrazolo[3,4-*d*]pyrimidine BK1369, also carrying a piperidinyl group, lacked antitumoral activity. These findings indicate that, although a piperidinyl group is optimal for PKD inhibition, it is not tolerated for cellular activity. Different mechanisms including metabolism, permeability and off-target effects could be responsible for these observations.

3. Conclusion

Based on our previously identified PKD inhibitor 3-IN-PP1, a novel series of pyrazolo[3,4-*d*]pyrimidines was synthesized. Systematic structural variation at position 1 of this scaffold led to the discovery of **17m**, which is the most potent pyrazolo[3,4-*d*]pyrimidine based pan-PKD inhibitor reported to date with IC_{50} values of 35, 35 and 17 nM for PKD1, 2 and 3, respectively. Despite the fact that compound **17m** was 3- to 5-fold more potent in a biochemical PKD assay, when compared to 3-IN-PP1, compound **17m** was less potent in cellular environments. On the other hand, 3-IN-PP1 very potently inhibited PKD activity in cells and blocked the proliferation of PANC-1 pancreatic cancer cells. Finally, a broad antitumoral screening revealed that 3-IN-PP1 is endowed with low-micromolar antiproliferative activity against 7 out of 8 cancer cell lines. Altogether, these data demonstrate that within the pyrazolo[3,4-*d*]pyrimidine based PKD inhibitors developed to date, 3-IN-PP1 clearly is the most potent antiproliferative agent.

Table 4. Inhibition of tumor cell growth of selected PKD inhibitors, data expressed as compound concentration required to inhibit tumor cell viability by 50% (IC₅₀).

Compound	IC ₅₀ (μM) ^a							
	LN-229	Capan-1	HCT-116	NCI-H460	DND-41	HL-60	K-562	Z-138
CRT0066101	0.6 ± 0.1	1.8 ± 0.1	1.7 ± 0.5	1.9 ± 0.2	1.0 ± 0.0	1.0 ± 0.4	2.2 ± 0.2	0.7 ± 0.3
1-NM-PP1	>100	23 ± 0.3	>100	>100	>26.1	24.1 ± 7	>36.3	>23.7
BKI1369	45.2 ± 6.8	>100	>55.2	>100	35.5 ± 19.7	>100	>100	30.4 ± 1.7
3-IN-PP1	39.2 ± 4.9	1.6 ± 0.4	2.5 ± 0.2	4 ± 0.8	9.8 ± 0.2	7.8 ± 0.7	3.7 ± 0.9	9.7 ± 3.5
13	>78.3	21.3 ± 9	63 ± 7.9	73.9 ± 22.7	>41.4	>25.6	>33.7	>38.2
17n	52.6 ± 0.4	4.7 ± 0.1	13.4 ± 0.7	14 ± 2.6	13.1 ± 0.6	27.6 ± 4.6	12 ± 0.9	19.6 ± 4.3
17m	80.6 ± 7	40.7 ± 14.5	>100	>95.8	42.7 ± 4.9	17.9 ± 4.1	>100	35.3 ± 10.4

^a Data are expressed as the mean ± SEM of n=2 independent experiments.

4. Materials and methods

4.1 Chemistry

Synthetic procedures, experimental details, spectral information (NMR spectra) are found in the supporting information of this article.

4.2 Biomolecular modelling

All homology modelling was performed using MOE2018 (Chemical Computing Group, Canada) with the Amber10-EHT force field. During the homology modelling process the crystallographically determined position of the ATP competitive inhibitors were retained for induced fit modelling. GOLD 5.7.2 was used for docking using the GoldScore and 100% search efficiency parameters.[42] The template ligands were used to determine the binding site with a radius of 6 Å. In order to prioritize the alternative binding mode, the nitrogens forming hydrogen bonds with the hinge served as pharmacophore features. Following docking, the binding modes and corresponding scores were sorted as classical, inverted and best energy. To evaluate the predictive value of the models, the Scikit-Learn Python library was used to calculate the ROC curves and AUC.[43] The residual activity cut-offs were chosen at 40, 50 or 60% to separate the active from inactive inhibitors.

4.3 Biological studies

4.3.1 Production and purification of recombinant FLAG tagged PKD1/2/3 in HEK293T cells.

HEK293T cells were cultured in DMEM (Sigma Aldrich, St. Louis, USA) supplemented with 1% Glutamax (Gibco, Life Technologies, California, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma Aldrich, St. Louis, USA) and 10% fetal bovine serum (Hyclone, GE Healthcare, Wisconsin, USA). Cells were plated at a density of 5 × 10⁶ cells per 10 cm plate and transiently transfected with 10 μg FLAG tagged pcdDNA-FLAG-PKD2 using polyethylenimine (PEI) (Polysciences Inc., Washington, USA) in a 5:1 (w/w) ratio. 48 hours after transfection cells were stimulated with 500 nM phorbol 12,13-dibutyrate (PDBu) (Sigma Aldrich, St. Louis, USA) for 15 minutes and subsequently lysed in a buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 50 mM NaF; 5 mM NaPPi (sodium pyrophosphate); 0.2 μM microcystine; 1 mM AEBSF (4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride); 10 μg/mL leupeptin; and 1 % triton X-100. Insoluble material was removed by centrifugation at 10000 × g for 15 minutes at 4 °C. Next, cell lysate was incubated with FLAG antibody beads (M2 Sigma Aldrich, St. Louis, USA) for 2 hours at 4 °C. The beads were washed 2 times with the aforementioned buffer but using 500 mM of NaCl instead of 150 mM. The proteins were eluted in 50 mM Tris pH 7.4, 50 mM NaCl, supplemented with 100 μg/mL FLAG peptide and stored in buffer containing 25% glycerol.

Protein concentration was determined via the Bradford assay using BSA as a standard and purity was determined by Coomassie stained SDS-polyacrylamide gel.

4.3.2 PKD screening assay

An *in vitro* luminescence based assay (ADP-Glo™ from Promega Corporation) was used to determine the PKD phosphorylation activity. Syntide-2 was used as a substrate and assays were performed in the presence of various compounds at a concentration of 1 μM for initial screening, and concentrations ranging from 10 μM to 1 nM for IC₅₀ determinations. Reactions were carried out in white 96-well flat bottom plates (NUNC) with a 10 μL reaction volume containing 50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 8 nM of PDBu stimulated FLAG PKD, 250 μM Syntide-2 (Sigma Aldrich, St. Louis, USA) and 10 μM ATP. After incubation of 15 minutes at 30 °C, the reaction was stopped and incubated with ADP-Glo reagent I and II (ratio 1:1:2, *i.e.* reaction volume: reagent 1: reagent 2, respectively) according to the manufacturer's protocol. Subsequently, the luminescence was measured using a Perkin-Elmer Victor X4 Multiple Plate Reader in counts per second (CPS). Percentage of PKD inhibition was determined relative to a DMSO control (0.1% DMSO). Graphs were plotted and IC₅₀ values were determined using the GraphPad Prism 7 software package.

4.3.3 Cell culture

PANC-1 cells were grown in Dulbecco's modified eagle medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (GE Healthcare, Little Chalfont, UK), 2 mM L-glutamine (Sigma, St. Louis, MO, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA).

4.3.4 Cellular screening assay using phospho-cortactin as a readout of PKD inhibition

PANC-1 cells were seeded at a density of 0.3 × 10⁶ cells per condition in a 6-well plate and allowed to attach. Twenty-four hours later, 0.5 μg of pcDNA-FLAG-PRKD1 or pcDNA-FLAG-PRKD2 and 0.5 μg of pcDNA3-Myc-CTTN were transfected using a PEI at a 1:3 (m/m) plasmid/ PEI ratio. After 24 hours, the cells were treated with the indicated compounds (Figure 3) at a final concentration of 20 μM or DMSO control for 1 hour, followed by either stimulation or no stimulation with 250 nM of phorbol-12,13-dibutyrate (PDBu) for 15 minutes. Lysis was done in 50 mM Tris, pH 7.4, 150 mM NaCl, 15 mM EDTA, 1% NP-40 supplemented with phosphatase inhibitors (Phosphostop, Roche, Germany), and protease inhibitors (cComplete, Roche, Germany). Protein quantification was determined using the BCA protein assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). Next, equal amounts of lysate (10 μg) were resolved in an SDS-PAGE and blotted into a nitrocellulose membrane. Antibody unspecific binding was blocked with either 5% BSA (Sigma, St. Louis, MO, USA) or 5% low-fat milk. Blots were probed with anti-Cortactin pSer-298 antibody (homemade),[31] anti-Myc 9E10 (Sigma, St. Louis,

Missouri) and anti-FLAG M2 (Sigma, St. Louis, Missouri). Finally, blots were incubated with the appropriate secondary HRP-linked antibody: Goat anti-Rabbit or Horse anti-Mouse (Cell Signaling Technologies Beverly, MA, USA). Band intensities were quantified with Image Studio Lite (LI-COR Biosciences). Ponceau staining was used as a loading control. The sharpness of the ponceau images was improved by using the default settings of the sharpen tool in Image Studio Lite software. Experiments were performed in triplicate (n=3). The statistical measures used were the mean and SEM. A one-way ANOVA was performed to determine the statistical significance with the control conditions (DMSO). Statistical analysis was conducted using the GraphPad Prism 7 software package.

4.3.5 CAMKII α and PKC δ assay

An *in vitro* radiometric assay was used to determine the inhibition of CAMKII α and PKC δ in presence of various compounds at the indicated concentrations. Reactions were carried out in 40 μ L of a mixture containing 50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 2 mM CaCl₂, 30 μ g/ μ L Calmodulin, 50 ng of CAMKII α (Carna biosciences, Kobe, JP), 2 μ g Syntide-2 (Sigma Aldrich, St. Louis, USA), 25 μ M ATP and 2 μ Ci [γ -³²P] ATP (Perkin-Elmer, Massachusetts, USA) for the CAMKII α assay. For the PKC δ assay, reactions were carried out in 40 μ L of a mixture containing 50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 4 μ L of phosphatidylserine (PS)/PDBu vesicles stock, 50 ng of PKC δ (Carna biosciences, Kobe, JP), 5 μ g MARCKS peptide (in-house synthesis), 25 μ M ATP and 2 μ Ci [γ -³²P] ATP (Perkin-Elmer, Massachusetts, USA). After 10 minutes, the kinase reaction was stopped by spotting 30 μ L of the reaction on Whatman P81 filter paper. The filter papers were washed three times with an aqueous solution of 0.5% phosphoric acid, followed by one wash with 100% acetone. Subsequently, the papers were air-dried and counted using a Tri-Carb 2810 TR scintillation counter (Perkin-Elmer). Percentage of inhibition was determined relative to a condition where no inhibitor was added (0.1% DMSO). Graphs were plotted using the GraphPad Prism 7 software package. PS/PDBu vesicles were prepared as follows: PDBu and PS were mixed to final concentrations of 100 μ g/mL PS and 250 nM PDBu and dried using a Savant Speedvac concentrator (Thermo Fisher Scientific, Waltham, MA, USA). Dried lipids were resuspended in 50 mM Tris pH 7.4 and sonicated three times for 10 minutes until a clear solution was achieved.

4.3.6 Proliferation test

PANC-1 cells were seeded at 2000 cells/well on a 96-well plate and allowed to attach for 4 hours. Subsequently, each compound was added to achieve a concentration of 5 μ M or DMSO (vehicle) was added to a final concentration of 0.25% as a negative control. Cells were allowed to grow for 0, 48, 96 and 144 hours before adding MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma, St. Louis, MO, USA) at final concentration of 0.5 mg/mL. The cells were incubated for 3 hours and thereafter lysed with DMSO. The absorbance at 550 nm was used as a readout of cell proliferation. Proliferation experiments were performed four times (n=4). The statistical measures used were the mean and SEM. A two-way ANOVA combined with Dunnett's multiple comparisons test was performed to determine the statistical significance with the control conditions (DMSO). Statistical analysis was conducted using the GraphPad Prism 7 software package.

4.3.7 Antitumor assays

All tumour cell lines were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA), except for the DND-41 cell line, which was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ Leibniz-Institut, Germany). All cell lines were cultured as recommended by the suppliers. Culture media were purchased from Gibco Life Technologies and supplemented with 10% fetal bovine serum (HyClone, GE Healthcare Life Sciences). Adherent cell lines LN-229, HCT-116, NCI-H460, and Capan-1 cells were seeded at a density between 400 and 1250 cells per well, in 384-well, black walled, clear-

bottomed tissue culture plates (Greiner). After overnight incubation, cells were treated with the test compounds at seven different concentrations ranging from 100 to 6.4x10⁻³ μ M. Suspension cell lines HL-60, K-562, Z-138, and DND-41 were seeded at densities ranging from 2500 to 5000 cells per well in 384-well, black walled, clear-bottomed tissue culture plates containing the test compounds at the same seven concentration points. The plates were incubated and monitored at 37 °C for 72 hours in an IncuCyte (Essen BioScience Inc., Ann Arbor, MI, USA) for real-time imaging. Images were taken every 3 hours, with one field imaged per well under 10 $\times$ magnification. All compounds were tested with duplicate data points and averaged.

Declaration of competing interests

The authors declare no competing financial interest

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