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REVIEW

Developments in the discovery and design of Protein Kinase D inhibitors

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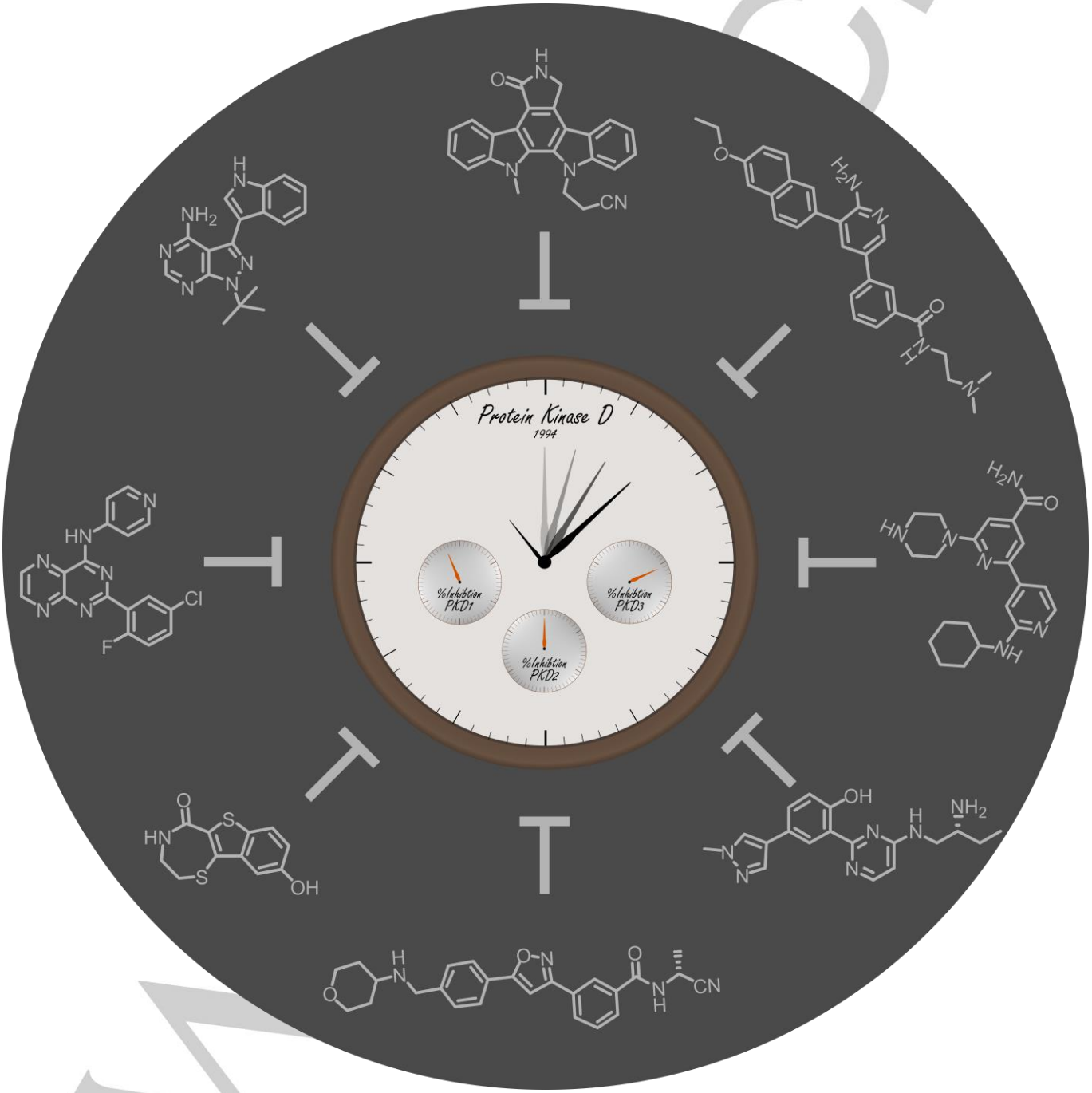

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Abstract: Protein kinase D (PKD) is a serine/threonine kinase family belonging to the Ca2+/calmodulin-dependent protein kinase group. Since its discovery two decades ago, many efforts have been put in elucidating PKD's structure, cellular role and functioning. The PKD family consists of three highly homologous isoforms: PKD1, PKD2 and PKD3. Accumulating cell-signaling research has evidenced that dysregulated PKD plays a crucial role in the pathogenesis of cardiac hypertrophy and several cancer types. These findings led to a broad interest in the design of small-molecule protein kinase D inhibitors. In this review, we present an extensive overview on the past and recent advances in the discovery and development of PKD inhibitors. The focus extends from broad-spectrum kinase inhibitors used in PKD signaling experiments to intentionally developed, bioactive PKD inhibitors. Finally, attention is paid to PKD inhibitors that have been identified as an off-target through large kinome screening panels.

1. Introduction

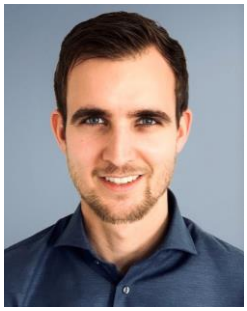
Protein kinase D (PKD) is a serine/threonine kinase family belonging to the CAMK kinase superfamily and gained considerable interest over the past two and a half decades.[1] Protein kinase D consists of three different isoforms, namely PKD1, 2 and 3 which are the subject of numerous cell signaling studies. These studies have ultimately led to the identification of PKD as an attractive drug target.[2] Insights in the role of protein kinases in cellular signal transduction mechanisms took a giant leap forward with the recognition that second messenger lipid diacylglycerol (DAG) binds and activates protein kinase C (PKC) enzymes.[3-4] Interestingly, with the discovery of the protein kinase D (PKD) family more than a decade later, it became clear that PKD's are targets for DAG as well.[1, 5-8] This led to a revision of the classical "textbook view" of signaling mechanisms, with a prominent role for the PKD's.

The three PKD's (PKD1, 2 and 3) share a common modular structure consisting from N- to C-terminal of: two DAG binding C1 domains, a pleckstrin homology (PH) domain and a catalytic kinase domain. While the catalytic domains of the three enzymes are highly homologous, their regulatory domains display considerable differences. Nevertheless, the three enzymes share a similar fundamental activation mechanism. It is assumed that in resting conditions, the PKD's exist in a "closed" state by tight interaction of the PH domain and the catalytic domain, thereby impairing its kinase activity. Upon receptor induced DAG production, both PKC's and PKD's are recruited to the plasma membrane where they bind DAG via their C1 domains.[9-11] There, the PKC's phosphorylate PKD on their activation loop, leading to full PKD activation.[12-13] The corresponding phosphorylation sites in the activation loop are Ser738/742 (PKD1), Ser706/710 (PKD2) and Ser731/735 (PKD3). PKD1 and PKD2 activation is often (but not always) accompanied by autophosphorylation at Ser910/Ser916 (human/murine) for PKD1 or Ser876/Ser873 (human/murine) for PKD2. This autophosphorylation event does not occur in PKD3 since it is lacking the corresponding site.[14-19]

Many stimuli are known to activate PKDs through this fundamental mechanism: growth factors acting through receptor tyrosine kinases (e.g. PDGF, IGF-I) and neuropeptide growth factors acting through G-protein coupled receptors (such as bombesin).[20-22] Besides this pathway, there is also an important pathway of PKD1 activation through oxidative stress. Here, oxygen radicals produced at the mitochondria cause a tyrosine phosphorylation in both the PH domain (by Abl) and the N-terminus (by Src), which in turn creates a docking point for PKCδ which phosphorylates and activates PKD1.[23-25] Furthermore, in the TGN part of the Golgi apparatus, PKD1 can become activated via interaction of G$\beta\gamma$ proteins with the PKD1 PH domain.[26] Together, these various mechanisms point to the PH domain as an important "hub" for different PKD activation mechanisms.

The above mentioned examples also indicate that PKD enzymes can be recruited to various cellular locations (plasma membrane, Golgi, mitochondria, nucleus…). Though it is not entirely clear yet how these different recruitments are regulated, it seems that local production of DAG, followed by C1 mediated PKD binding could play a crucial role besides the interaction with adaptor proteins such as AKAPs.[27-28] Over the years, many substrates of the PKD's have been discovered, and this has allowed a clearer positioning of the PKD's on the cellular signaling map, both in health and disease.

The discovery of PKD1's role in the pathogenesis of cardiac hypertrophy formed the initial impetus in pharma as well as in academia for the development of PKD inhibitors.[29-30] In response to pathologic stresses such as myocardial infarction or hypertension, a remodeling process takes place in the heart, leading to cardiomyocyte hypertrophy and fibrosis, resulting in reduced cardiac functioning and ultimately heart failure.[31-32] PKD1 was found to be a master regulator of this process, via its phosphorylation of histone deacetylases HDAC4 and 5, thereby unleashing the expression of cardiomyocyte genes that contribute to the above described disease progression.[29, 33] Several preclinical studies in cellular and mouse models have indicated a beneficial effect of PKD inhibitor treatment on heart hypertrophy.[34-35]



Philippe Gilles obtained his BSc and MSc degrees in Chemistry at KU Leuven. In December 2016, Philippe was granted an FWO SB scholarship enabling him to conduct doctoral research on the development of PKD inhibitors in the research group of Professor Wim De Borggraeve (KU Leuven). In fall 2018, Philippe spent five months in the cheminformatics group of Prof. Thierry Langer (University of Vienna), working on virtual screening and chemical modeling. In November 2020, Philippe obtained his PhD in Chemistry at KU Leuven.

Lauren Voets received her BSc degree in Chemistry with a minor in biochemistry and biotechnology in 2017 and graduated as MSc in Chemistry in 2019 at KU Leuven. During her master thesis, she developed novel anti-seizure compounds, under the supervision of Prof. Wim De Borggraeve. In November 2020, Lauren started her PhD in the same group as a Strategic Basic Research fellow funded by the FWO. Her research is focused on the design, synthesis and evaluation of Protein Kinase D inhibitors.

Johan Van Lint obtained his MD and PhD degrees at KU Leuven in 1989 and 1993 respectively. From 1993-95 he was an EMBO postdoctoral fellow at the Imperial Cancer Research Fund (now CRUK division, Francis Crick institute, London). In 2004 he was appointed as Professor at KU Leuven and now leads the Laboratory for Protein Phosphorylation & Proteomics at the department of Cellular & Molecular Medicine. His research interest is focused on the Protein Kinase D family and its role in health and disease.

Wim De Borggraeve obtained his PhD in Chemistry at KU Leuven in 2002. After a postdoc with FWO Flanders and research stays in the groups of W.D. Lubell (Université de Montréal, Canada) and C. Toniolo (University of Padova, Italy), he was appointed at KU Leuven in 2009 where he currently holds a Professor position. His research interests are in organic synthesis methodology, heterocyclic and medicinal chemistry.

Next to cardiac hypertrophy, increasing evidence suggests an important role of PKD1 as a central nutrient-sensing regulatory kinase involved in the maladaptation to excess lipid accumulation in many tissues (fat, skeletal muscle, heart, liver).[36] This would suggest that PKD1 inhibition could be beneficial in a setting of obesity treatment. However, since PKD1 is also required in the pancreas for glucose stimulated insulin secretion, such an approach could be problematic.

Another research area that provoked strong interest in the development of PKD inhibitors is cancer. Over the past decades, the gradual elucidation of the so-called “hallmarks of cancer” has provided drug developers with a framework to develop anticancer drugs that target key proteins involved in these hallmarks.[37-38] Remarkably, the PKD enzymes are involved in the regulation of several of these hallmark properties, such as proliferation, apoptosis dysregulation, metastasis and angiogenesis.[1, 8, 39-40] However, the effect of the three PKDs on these hallmarks is very different. Indeed, whereas PKD2 (and to a large extent also PKD3) most consistently promote the development of these hallmarks, the PKD1 isoenzyme very often counteracts the development of the above-mentioned hallmarks.[1, 8, 39-40] However, caution is required not to over-generalize these notions, as these isoenzyme properties are highly tumor type specific.[8]

At present, the role of the PKD’s in cancer has been most intensively studied in pancreatic cancer,[41] breast cancer [42] [43-44] and prostate cancer.[45-47] As a consequence several PKD inhibitor molecules have been developed that were subsequently shown to inhibit cancer growth in cellular models, xenograft mouse models and orthotopic mouse models for pancreatic cancer,[48-49] breast cancer[50-51] and prostate cancer.[52-54] The purpose of this review is to provide an overview of current knowledge regarding PKD inhibitors that have been discovered over the past decades and which in general can be subdivided in three classes: broad-spectrum kinase inhibitors, inhibitors optimized towards PKD and inhibitors that hit PKD as an off-target.

2. Protein kinase D inhibitors

The first part of this text discusses early-discovered PKD inhibitors, including several staurosporine analogues, which usually inhibit a broad-spectrum of kinases. Many of these inhibitors have mainly been used right after the discovery of PKD to study its role in cellular signal transduction pathways. Secondly, inhibitors that resulted from PKD-oriented drug discovery campaigns will be discussed. Most of these compounds have been identified via high-throughput screening followed by structural optimization. Finally, inhibitors will be discussed that have been part of large kinome screening studies and exhibit off-target activity towards PKD.

2.1. Broad-spectrum kinase inhibitors

Staurosporine & derivatives thereof

The archetypal natural kinase inhibitor is staurosporine (Figure 1). The natural product was discovered in 1977 at the Japanese Kitasato Research Institute in Japan where it was isolated from *Streptomyces staurosporeus*.[55] Years later, it was found that staurosporine is endowed with low-nanomolar inhibitory activity against protein kinase C.[56] As a mimic of ATP, it fits in the adenosine pocket located at the interface of the N- and C-lobe of protein kinases. Large screening campaigns, however, demonstrated that staurosporine inhibits more than 70% of the human kinome.[57] PKD was not an exception in this study as staurosporine shows low-nanomolar inhibitory activity against all three isoforms of PKD (K_d = 18-72 nM for PKD1-3).

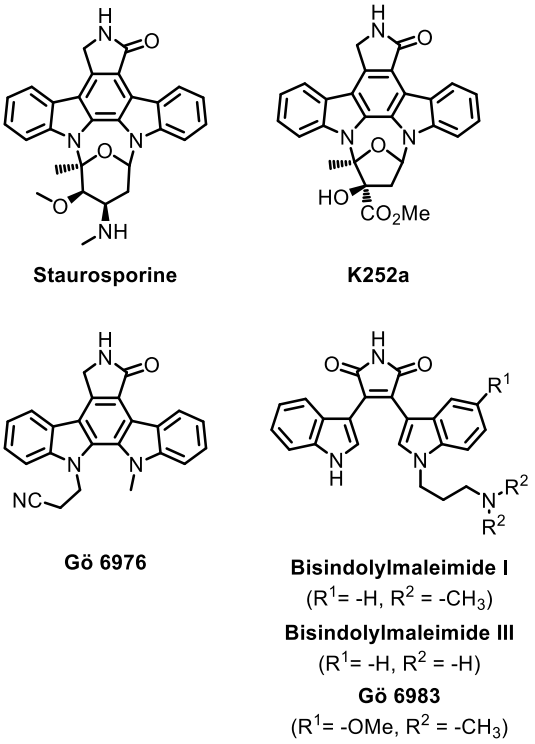


Figure 1. Structure of natural product staurosporine and derivatives thereof.

Several structurally related analogues of staurosporine have demonstrated similar potency against PKD (Figure 1 and Table 1).[58] Interestingly, highly potent PKC inhibitors K252a, Gö 6976, Gö 6983 and bisindolylmaleimides I & III show different PKD/PKC selectivity profiles. While Gö 6976 is a strong inhibitor of both the PKC family and PKD1, the bisindolylmaleimides and Gö 6983 are about 100 to 1000-fold less potent against PKD1. These results indicate that the central aromatic core is crucial for strong PKD1 inhibition. Furthermore, the reduced PKD inhibitory potency of PKC inhibitor Gö 6983 has provided researchers with a tool to investigate dependency of PKC in PKD signaling.[59-61]

Table 1. Overview of staurosporine derived PKD inhibitors.

Compound	IC_{50} PKD1 (nM)
staurosporine	40
K252a	7
GÖ6976	20
GÖ6983	20 000
bisindolylmaleimide I (GF109203X)	2 000
bisindolylmaleimide III	10 000

PKA inhibitor H-89

In 1990, isoquinolinesulfonamide H-89 was discovered as a potent protein kinase A (PKA) inhibitor. H-89 showed only weak off-target inhibition against various other protein kinases and was thus considered as a potent, selective PKA inhibitor with a K_i-value of 0.048 µM.[62] A few years later, however, a screening of several known protein kinase inhibitors against PKD1 revealed isoquinoline-sulfonamide H-89 as a moderate PKD1 inhibitor (Figure 2). By means of dose-response curves, the IC_{50} value for H-89 was estimated at 0.5 µM.[63] Follow-up profiling studies have identified more kinase off-targets and therefore its utility as specific PKD inhibitor is limited.[64] Nonetheless, H-89 has been used several times in research studying PKD1-related cell signaling.[65-67]

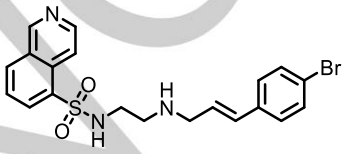


Figure 2. Structure of PKA inhibitor H-89 with off-target PKD activity

Resveratrol

Another broad-spectrum agent that has been reported to inhibit PKD is resveratrol (Figure 3).[68] Resveratrol is a known anti-tumorigenic natural product influencing various stages of carcinogenesis.[69] Given the inhibitory potency of resveratrol to phorbol ester-mediated tumor promotion, its antagonism against phorbol ester-responsive PKCs and PKD has been evaluated. Resveratrol is a weak inhibitor of PKD1 with IC_{50} values of ± 36 µM, but does not affect the tested PKCs.[68] Further studies have shown that very high concentrations of resveratrol are required to induce PKD inhibition in cellular settings (IC_{50} = ± 800 µM).[70] Resveratrol more potently inhibits other targets (including cyclooxygenases and other kinases) at lower concentrations which compromises its utility as a pharmacological PKD inhibitor.[69]

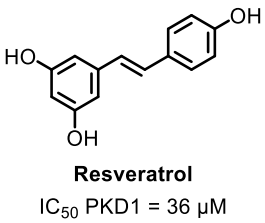


Figure 3. Structure of weak PKD inhibitor resveratrol.

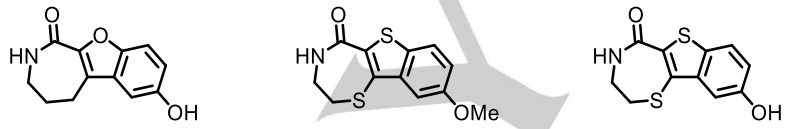
2.2. Inhibitors resulting from PKD-oriented drug discovery campaigns

2.2.1. Cancer

CID755673

The first series of PKD inhibitors that were intentionally designed to inhibit PKD are the benzoxoloazepinolones. By means of a high-throughput, immobilized metal affinity for phosphochemicals (IMAP)-based fluorescence polarization screening assay (Pubchem AID 797), CID755673 was identified as a potent PKD1 inhibitor (Figure 4).[71] Interestingly, this compound inhibits all three isoforms of PKD to the same extent in a non-ATP dependent fashion. The exact binding mechanism, however, remains elusive to date. Further characterization demonstrated that CID755673 exhibits sub-micromolar activity against PKD1 (IC_{50} = 182 nM) with a notable selectivity profile; in contrast to many previously described inhibitors, CID755673 did not inhibit the related PKC family. Inhibition of PKD1 Ser^{916} autophosphorylation in LNCaP prostate cancer cells demonstrated that CID755673 was also active in cells. In addition, the authors showed that this compound inhibits cell migration, invasion and proliferation of various prostate cancer cell lines (LNCaP, PC3 and DU145) with IC_{50}'s in the low double-digit micromolar range. CID755673 was profiled against a panel of 46 kinases at a concentration of 10 µM and showed an inhibition percentage ≥ 50% for six other kinases (CDK2, CK1δ, ERK1, GSK3β, MAPKAPK2 and MK5/PRAK).[52] The full selectivity profile of this compound class, however, remains unclear and PKD1-independent biological effects of CID755673 have been observed.[72]

Following up on the discovery of CID755673, further SAR studies have been conducted leading to analogues kb-NB165-09 and kb-NB142-70 which were respectively two- and almost seven-fold more potent (Figure 4).[73-74] Furthermore, derivative kb-NB142-70 was found to have increased potency towards inhibiting prostate cancer cell proliferation (PC3 cells), migration (PC3 and DU145 cells) and invasion (DU145 cells).[52, 73] Furthermore, the compound also inhibits the growth of pancreatic cancer cells (PANC-1 and CFPAC-1).[49] Subsequent studies investigated the cytotoxicity and both the pharmacokinetics and pharmacodynamics of kb-NB142-70 in-depth. Unfortunately, kb-NB142-70 is rapidly metabolized after administration and was not active at the maximum soluble dose in xenograft mice.[49] Nevertheless, CID755673 and kb-NB142-70 nowadays serve as valuable tools for biochemical studies on PKD signaling. Notable examples are the studies on the role of PKD as regulator of necrosis in pancreatic acinar cells and its role in wound-induced migration of intestinal epithelial cells.[75-77]



CID755673
IC_{50} PKD1 = 182 nM
IC_{50} PKD2 = 280 nM
IC_{50} PKD3 = 227 nM

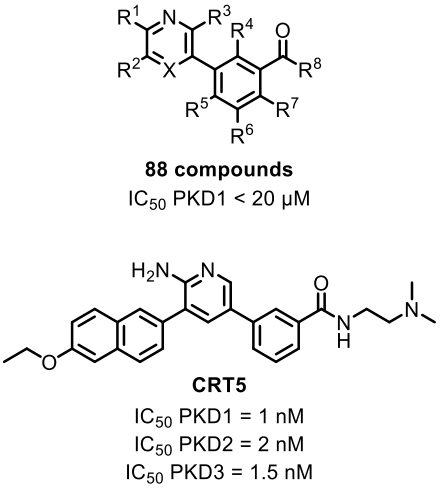
kb-NB165-09
IC_{50} PKD1 = 83 nM
IC_{50} PKD2 = 142 nM
IC_{50} PKD3 = 99 nM

kb-NB142-70
IC_{50} PKD1 = 28 nM
IC_{50} PKD2 = 59 nM
IC_{50} PKD3 = 53 nM

Figure 4. Structure and biochemical activity of benzoxoloazepinolone CID755673 and potent analogues thereof.

CRT5

In an effort to study the role of PKD in VEGF-mediated angiogenesis, a high-throughput screening was conducted against PKD1 starting from approximately 55 000 compounds. Within this library, multiple pyridine benzamides and pyrazine benzamides demonstrated potent PKD inhibitory activity.[78] Ultimately, lead compound CRT5 was identified as a strong PKD inhibitor with biochemical IC_{50} values of 1, 2 and 1.5 nM against PKD1, 2 and 3 respectively (Figure 5).[79] Interestingly, CRT5 had little biochemical inhibitory activity against various PKC enzymes. Additional studies demonstrate that CRT5 is also active in cellular settings. The compound is capable of blocking autophosphorylation of PKD1 and 2 (at Ser^{916} and Ser^{876}, respectively) and phosphorylation of downstream substrates including HSP27, CREB and HDAC5. In addition, the authors demonstrated that CRT5 is capable of reducing VEGF-stimulated migration and VEGF-induced tubule formation; both mechanisms associated with endothelial cell angiogenesis. Furthermore, the observed data was in agreement with earlier PKD siRNA knockdown studies.[80] In a subsequent study on the function of PKD in human rhinovirus replication, CRT5 showed a promising kinome selectivity profile. Out of the 127 tested kinases, only 5 off-targets (BMX, BRK, cKIT(V560G), EGFR(T790M) and VEGFR1) were inhibited more than 50% at a 1 µM concentration.[81]



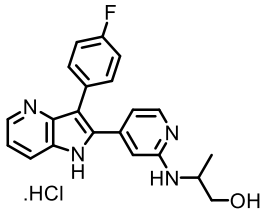
88 compounds
IC_{50} PKD1 < 20 µM

CRT5
IC_{50} PKD1 = 1 nM
IC_{50} PKD2 = 2 nM
IC_{50} PKD3 = 1.5 nM

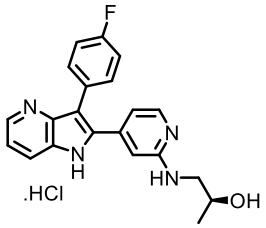
Figure 5. Structure of pyridine benzamides, pyrazine benzamides and amino pyridine analogue CRT5.

Compound 139, 140 and 209

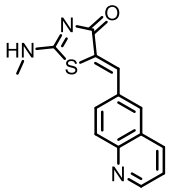
During a screening of a small kinase inhibitor library, Tandon *et al.* identified several novel PKD inhibitors.[82] Within this set, six small molecules were obtained having favorable selectivity over related PKC (PKCα & PKCδ) and CAMK (CAMKIIα) kinases (inhibition less than 50% at 1 µM). The authors found IC_{50}-values of 17 and 562 nM for compounds 139 and 209, respectively (Figure 6). Both compounds were assessed for their inhibitory effect on PKD autophosphorylation at Ser^{916} in LNCaP prostate cancer cells. Following the biochemical data, cellular IC_{50}-values of 1.5 µM for compound 139 and 18 µM for compound 209 were determined. The selectivity of compound 140, a closely related derivative of compound 139 which is equally active, was assessed in a kinome-wide screening against 353 kinases. At a concentration of 10 µM, 43 kinases were inhibited more than 50% while only 8 kinases were completely inhibited.



Compound 139
PKD1 inhibition (1 µM) = 94%
IC_{50} PKD1 = 17 nM



Compound 140
PKD1 inhibition (1 µM) = 83%
PKD2 inhibition (1 µM) = 99%
PKD3 inhibition (1 µM) = 96%

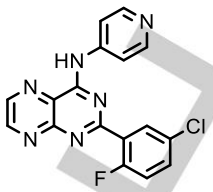


Compound 209
PKD1 inhibition (1 µM) = 64%
IC_{50} PKD1 = 562 nM

Figure 6. Structure of compounds 139, 140 and 209.

SD-208

Screening of the Tocriscreen kinase inhibitor collection containing 80 kinase inhibitors revealed the *in vitro* potency of SD-208 as PKD inhibitor.[54] SD-208 inhibits all three PKDs with IC_{50} values of 107, 94 and 105 nM (Figure 7). Furthermore, the compound is competitive with ATP and proven active in cellular assays. Although structural variation was introduced on the pteridine scaffold, the SAR analysis did not result in inhibitors that were significantly more potent. The study also demonstrated that 30 µM of SD-208 was capable of significantly reducing cell proliferation and invasion of prostate cancer cells. The authors do not exclude, however, that the latter effect could also be ascribed to the fact that SD-208 is also an inhibitor of the Transforming Growth Factor β Receptor I (TGFβR-I). Finally, administration of SD-208 reduced tumor growth in a prostate cancer xenograft mouse model. After oral administration for 11 days, a statistically significant reduction in tumor volume was observed in comparison to the control group.

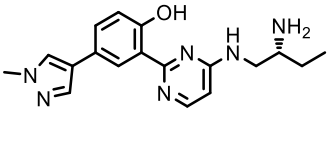


SD-208
IC_{50} PKD1 = 107 nM
IC_{50} PKD2 = 94 nM
IC_{50} PKD3 = 105 nM

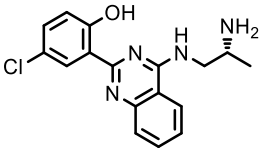
Figure 7. Structure of pteridine SD-208.

CRT0066101

After screening a diverse compound library, Guha and co-workers discovered CRT0066101 as a strong pan-PKD inhibitor (IC_{50} = 1, 2.5 and 2 nM for PKD1, 2 and 3, respectively) exhibiting remarkable anti-cancer properties in cells and animals (Figure 8).[48, 83] CRT0066101 significantly inhibits proliferation of Colo357, PANC-1, MiaPaCa-2 and AsPC-1 pancreatic cancer cell lines, all expressing moderate to high levels of endogenous PKD1 and 2. Furthermore, treatment of PANC-1 cells with CRT0066101 increases apoptosis as was demonstrated by increased caspase-3 activity. In addition, treatment of both hetero- and orthotopic PANC-1 xenograft mice with CRT0066101 leads to a significant tumor growth reduction. In follow-up studies, the same authors also investigated the effects of CRT0066101 on cell proliferation in both PKD1 overexpressing PANC-1 cells and high PKD1 expressing PANC-28 cells.[84] In both cases, the compound significantly reduces cell proliferation and invasion. Furthermore, CRT0066101 reduces angiogenesis *in vitro* by inhibiting the secretion of VEGF and CXCL8 chemokines. This reduction was further analyzed using orthotopically implanted PANC-1 tumors. CRT0066101 significantly attenuates microvessel formation in treated mice thereby effectively blocking tumor angiogenesis. In subsequent studies on experimental pancreatitis in rat acini, it has been demonstrated that CRT0066101 is capable of inhibiting early events of acute pancreatitis, presumably through the promotion of apoptosis and attenuation of necrosis.[75-76, 85] The latter is an uncontrolled form of cell death, which releases intracellular constituents to extracellular space and correlates with increased severity of pancreatitis.



CRT0066101
IC_{50} PKD1 = 1 nM
IC_{50} PKD2 = 2.5 nM
IC_{50} PKD3 = 2 nM



XX-050
IC_{50} PKD1 = 1.6 nM

Figure 8. Structure of PKD inhibitor CRT0066101 and closely related derivative XX-050.

CRT0066101 shows also promising activity in various colorectal cancer models including the HCT-116, RKO, H630 and H630R1 cell lines and in HCT-116 mice xenografts.[86] Further analysis showed that CRT0066101 induces pro-apoptotic responses in RKO and HCT-116 cells after treatment. Given this promising potency against colorectal cancer, the combination treatment of CRT0066101 with regorafenib has been investigated.[87] The latter is an FDA-approved multi-kinase inhibitor for the treatment of metastatic colorectal cancer. The authors found that the combination of regorafenib and CRT0066101 exerts synergistic effects on the *in vitro* growth inhibition of colorectal cancer cells. The combination of both agents induces increased apoptosis and increased inhibition of the RAS/RAF/ERK, PI3K/AKT/mTOR, and NF-κB signaling pathways.

The effects of CRT0066101 have also been studied in several other cancer models. Pharmacological inhibition of PKD2 in glioblastoma cells decreases glioma cell proliferation, migration and invasion.[88-89] Similar *in vitro* results have been observed in breast cancer models and CRT0066101 demonstrates efficacy in reducing tumor growth of estrogen receptor negative (HCC1954), multidrug-resistant (MCF-7-ADR) and triple negative breast cancer (MDA-MB-231 & MDA-MB-468).[50-51, 90] For the latter, CRT0066101 also suppressed tumor growth in xenograft mouse models. Finally, a recent study described the use of CRT0066101 in the treatment of bladder cancer. The compound effectively blocks bladder cancer cell proliferation, anchorage independent growth, migration and invasion in various cell lines (T24, T24T, TCCSUP, and UMUC1).[91] Furthermore, CRT0066101 significantly reduces tumor growth of subcutaneously implanted UMUC1 cells in mice.

In a study on the use of PKD inhibitors for the inhibition of viral replication, another closely related, potent derivative has been disclosed.[81] Compound XX-050 shares the same 2-(4-aminopyrimidin-2-yl)phenol central scaffold while the *N*-methylpyrazolyl moiety is replaced with a chlorine substituent and the 2-aminobutyl chain with a 2-aminopropyl chain (Figure 8). CRT0066101, XX-050 and CRT5 all potently inhibited replication of picornaviruses, presumably through the inhibition of PKD. In a kinome selectivity screening, CRT0066101, XX-050 and CRT5 have been tested against a panel of 127 kinases and, between these molecules, no off-targets that were significantly overlapping were found. These data further indicate the potential role of PKD in viral replication. The panel also revealed that CRT0066101 shows more than 50% inhibition at 1 µM of 13 other kinases (including various CDK's, DYRK2 and Pim-1) while XX-050 blocked 38 kinases. Although CRT0066101 is usually used as a "PKD specific" inhibitor in cell biology, these data indicate that attention should be paid to potential, non-innocent off-target effects.

Pyrazolo[3,4-*d*]pyrimidines

Another important class of PKD inhibitors is derived from pyrazolo[3,4-*d*]pyrimidine 1-NM-PP1. This compound was originally designed by the group of Shokat as a specific inhibitor of engineered analog-sensitive (AS) v-Src protein kinases.[92] These modified kinases are "programmed" to recognize specific inhibitors that do not inhibit wild-type kinases. Most protein kinases have a medium to large sized gatekeeper residue in the ATP binding pocket that blocks access to a hydrophobic back pocket (Figure 9B). In an analog sensitive kinase, this gatekeeper is mutated *a priori* to a smaller residue allowing the naphthalene moiety of 1-NM-PP1 to access this back pocket (Figure 9C)

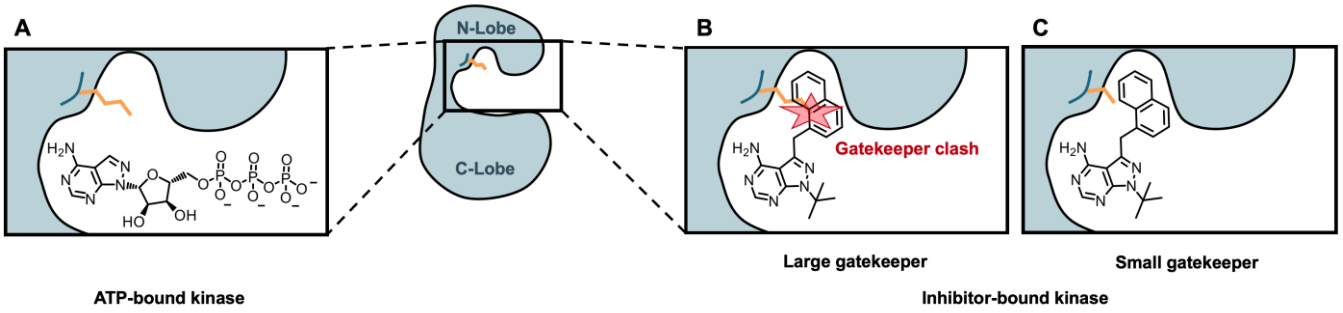


Figure 9. Principle of inhibitors sensitive towards gatekeeper mutations. (A) ATP binding to the active site of a kinase. (B) 1-NM-PP1 clashes with large gatekeeper residues. (C) 1-NM-PP1 binds to the modified kinase with a smaller gatekeeper.

Despite this well-established technology, 1-NM-PP1 and its close derivative 1-NA-PP1 do have non-mutated protein kinase off-targets, including wild-type PKD.[53, 64, 93] Elaborating on these findings, our group previously discovered that replacement of the naphthalene moiety of 1-NM-PP1 with an indole substituent (3-IN-PP1) further increases the inhibitory potency against PKD by a factor of ten (Figure 10).[94] Unfortunately, other synthetic efforts focusing on both position 1 and 6 did not result in more potent compounds. 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. Ultimately, compound **17m** was found as the most potent pyrazolo[3,4-*d*]pyrimidine reported to date.[95] PKD inhibitor **17m** along with 3-IN-PP1 were evaluated for their cellular activity. By monitoring PKD specific cortactin phosphorylation, both compounds were found to clearly inhibit cellular PKD activity. When evaluating the same set of compounds in a cell proliferation assay, 5 µM of 3-IN-PP1 significantly decreased pancreatic cancer cell (PANC-1) proliferation. From these data, it also appeared that despite its very potent *in vitro* activity, **17m** has

lower *in cellulo* potency. The exact reasons, however, are not yet clear and might be a result of increased metabolism, lower cell permeability or off-target activity. Finally, a global anti-tumorigenic screening revealed that 3-IN-PP1 has potencies similar to CRT0066101 against a series of eight other 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).

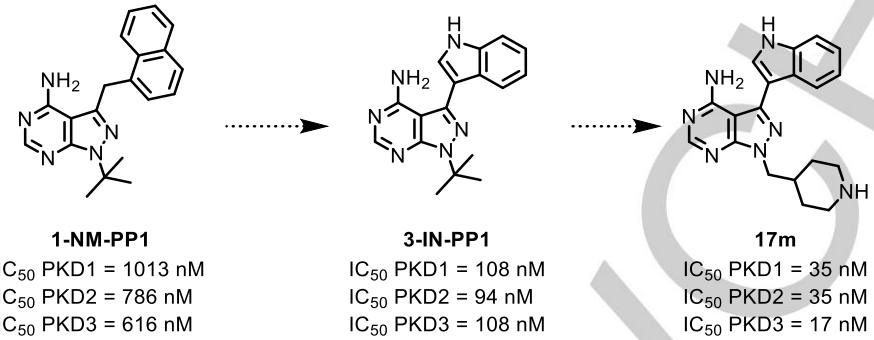


Figure 10. Optimization of pyrazolo[3,4-*d*]pyrimidine-based PKD inhibitors.

Other PKD inhibitors

Sharlow and co-workers published a series of PKD inhibitors that has been identified during their HTS campaigns.[96] Apart from CID 755673, several other analogues were discovered and have been studied for their *in vitro* inhibitory potency against PKD1 with biochemical IC_{50} values ranging from 0.4 to 6.1 µM. Three of them, namely CID 2011756, CID 5389142 and CID 1893668, have cellular activity: they potently inhibit phosphorylation of endogenous PKD1 at Ser^{916} in LNCaP prostate cancer cells (Figure 11). Unlike CID 755673, these three inhibitors all inhibit PKD in an ATP competitive fashion.

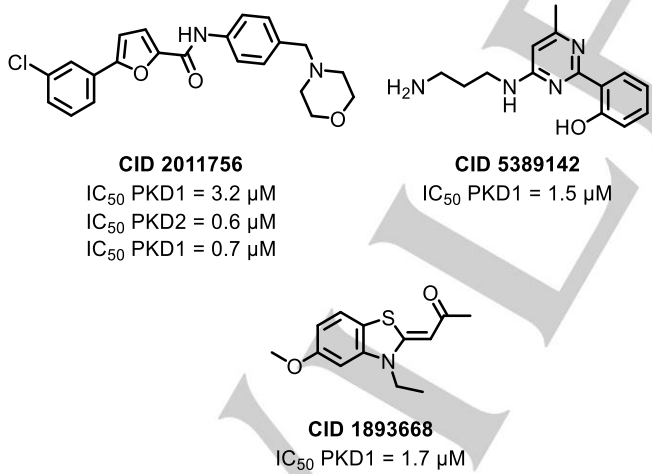


Figure 11. Structure of CID 2011756, CID 5389142 and CID 1893668.

Very recently, researchers at Genentech published a series of substituted propargylic alcohols as potent PKD1 inhibitors.[97] During their earlier studies on novel NF-κB Inducing Kinase (NIK) inhibitors, the authors found that the alkyne moiety presumably has a beneficial role in inhibiting methionine gatekeeper kinases as it can extend beyond this gatekeeper residue.[98] In this way, the alcohol is presented in the back pocket allowing specific hydrogen bond interactions. In a broad screening of this compound class against a panel of protein kinases, off-target activity against protein kinase D was detected. In a subsequent study, the propargylic alcohols such as **(*R*)-2** were further optimized towards PKD1-selective inhibitors (Figure 12).[97] It was found that the alcohol function and its stereochemistry were important features required for potent NIK inhibition whilst not for PKD1 inhibition. Therefore, inversion of the chiral center and introduction of a cyclopropyl group on the imidazole ring resulted in high affinity PKD1 inhibitor **(*S*)-12** that completely lost potency against NIK. Finally, **(*S*)-12** was screened against 220 other kinases at a concentration of 0.1 µM and interestingly no off-targets were inhibited by more than 30%.

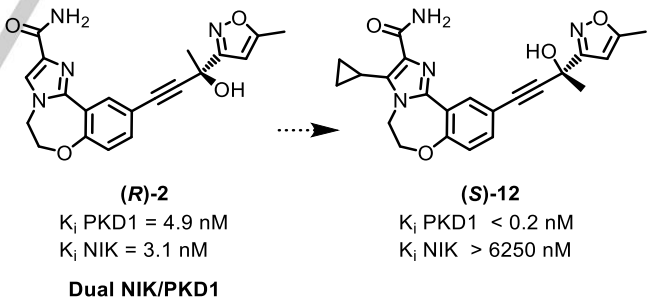


Figure 12. Optimization of dual NIK/PKD1 inhibitors towards PKD1 selective inhibitors.

2.2.1. Cardiac hypertrophy

Bipyridines and 2,6-naphthyridines

PKD has been reported to be an upstream mediator of class IIa HDAC nuclear export, a process that mediates stress-induced cardiac hypertrophy.[99] In an effort to study the application potential of PKD inhibitors in heart failure therapy, researchers at the Novartis Institutes for BioMedical Research performed a high-throughput activity screening of >650 000 compounds against full-length PKD1. During this screen, a pyridine-substituted 2,6-

naphthyridine (naphthyridine **2**) was identified as a potent dual PKD/PKC inhibitor (Figure 13).[35] Notwithstanding its nanomolar activity against PKD, this molecule was even more potent against PKC. The latter, however, is a direct upstream activator of PKD and therefore an inhibitor with selectivity over PKC was desired. Through substantial optimization of the hit molecule, more potent cyclohexylaminopyridine-substituted naphthyridine **13c** was obtained displaying low-nanomolar activity against PKD1 (IC_{50} = 0.6 nM) and a 1000-fold selectivity over PKCα and PKCδ (IC_{50} = 1000 nM and 881 nM, respectively). Despite this high potency, naphthyridine **13c** lacked the desired selectivity over other kinases to clearly study the role of PKD in cardiac myocyte stress response. Therefore, a second round of optimization was performed yielding bipyridine inhibitor BPKDi with increased kinase selectivity.[34, 100] Both the naphthyridine and bipyridine PKD inhibitors reduced autophosphorylation of PKD and downstream HDAC phosphorylation in cells. Despite its very potent activity, BPKDi ultimately could not reduce cardiac hypertrophy in animal models.[100]

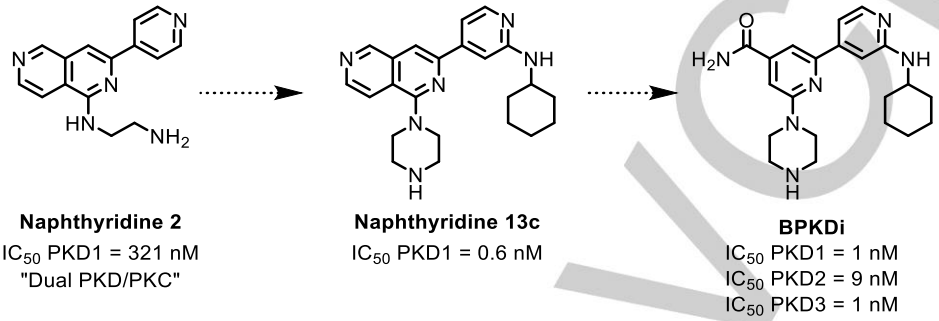


Figure 13. Structure of 2,6-naphthyridine PKD inhibitors and structurally related bipyridine BPKDi.

3,5-diarylisoxazoles

During their research on the applicability of PKD inhibitors for the treatment of cardiac hypertrophy, Novartis also pursued another class of molecules. The 3,5-diarylpyrazole hit compound exhibits moderate inhibitory potency (IC_{50} = 2.3 µM) against PKD1 (Figure 14).[101] Thorough optimization revealed that replacement of the pyrazole core with an isoxazole and introduction of an *R*-α-aminopropionitrile amide substituent together with a tetrahydropyranyl-substituted benzyl amine were crucial to potently inhibit PKD (IC_{50} = 5, 48 and 17 nM for PKD1, 2 and 3, respectively). The compound is also capable of blocking HDAC5 nuclear export (EC_{50} = 0.24 µM) in cells and has good *in vitro* metabolic stability. Finally, the authors assessed this compound in a panel of 230 kinases finding only 4 off-targets which were inhibited by more than 50% at 1 µM.

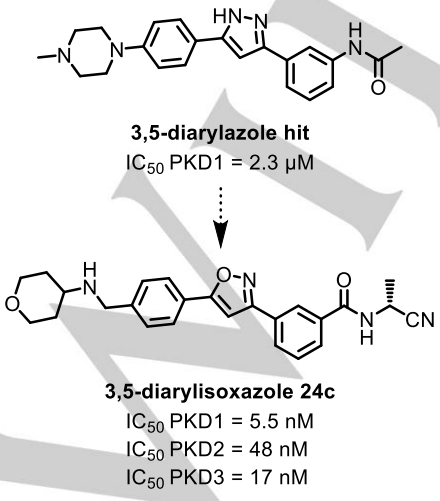
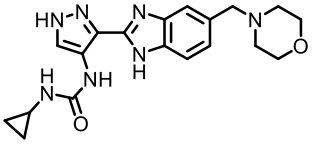


Figure 14. Structure of the 3,5-diarylazole hit compound and the optimized PKD inhibitor 3,5-diarylisoxazole 24c.

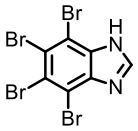
2.3. Off-target PKD inhibitors identified in drug discovery campaigns and large kinome profiling studies

Since the successful development of imatinib, the first marketed protein kinase, many drug discovery campaigns have oriented towards this class of enzyme targets. However, one of the main hurdles in kinase inhibitor development is designing molecules that exhibit selectivity over other kinases in the human kinome, mitigating potential undesired off-target effects. Therefore, many drug discovery campaigns typically screen their lead compounds towards a panel of kinases to examine their selectivity profiles. Such kinome profiling studies have led to several off-target PKD inhibitors which will be discussed here.

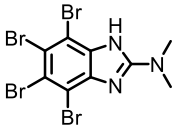
In a study on the development of dual aurora kinase inhibitors, pyrazol-4-yl-urea AT9283 (Figure 15) was identified as an off-target PKD inhibitor.[102-103] A counter-screen against > 144 kinases identified the potential of the urea-based compound as a multitarget inhibitor against JAK2 (IC_{50}: 1.2 nM), Abl (IC_{50}: 4 nM) and PKD1 (IC_{50}: 10-30 nM).[102] Next to these pyrazol-4-yl-urea derivatives, benzimidazoles TBI and DMAT show off-target activity towards PKD1 (Figure 15). These benzimidazole compounds were originally described as potent and selective CK2 inhibitors. However, taking a closer look at their activity against 80 different kinases revealed significant off-target effects to several other kinases.



AT9283
IC_{50} CDK2 = 510 nM
IC_{50} Dual Aurora A/B= 3 nM
IC_{50} PKD1 = 10-30 nM



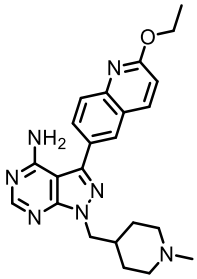
TBI
IC_{50} CK2 = 0.60 µM
IC_{50} PKD1 = 0.34 µM



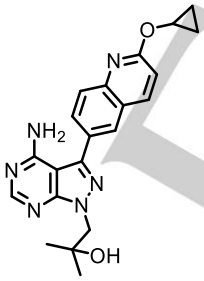
DMAT
IC_{50} CK2 = 0.13 µM
IC_{50} PKD1 = 0.18 µM

Figure 15. Off-target identified PKD inhibitors AT9283, TBI and DMAT.

Interestingly, off-target PKD activity has also been noted in a chemoproteomic profiling study of BKI-1369 and BKI-1649. This compound is a pyrazolopyrimidine-based bumped kinase inhibitor (BKI) developed to target parasitic kinase *Toxoplasma gondii* calcium-dependent protein kinase 1 (*Tg*CDPK1). However, in a kinobead screening towards approximately 200 kinases, BKI-1369 and BKI-1649 were found to potently block PKD with IC_{50} values in the range of 31-140 nM (Figure 16).[104]



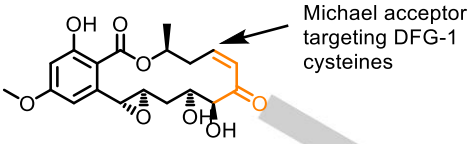
BKI-1369
IC_{50} PKD1 = 140 nM
IC_{50} PKD2 = 43 nM
IC_{50} PKD3 = 54 nM



BKI-1649
IC_{50} PKD1 = 67 nM
IC_{50} PKD2 = 31 nM
IC_{50} PKD3 = 31 nM

Figure 16. Structure and off-target PKD activity of bumped kinase inhibitors BKI-1369 and BKI-1649.

Despite the progress on the target-oriented design of reversible PKD inhibitors, little to no progress has been made on the rational design of covalent, irreversible PKD inhibitors. To the best of our knowledge, only one irreversible PKD inhibitor is known, hypothemycin, which has been identified through a kinome profiling study.[105] Based on structural data of hypothemycin in complex with ERK2, it is presumed that hypothemycin would bind to PKD's DFG-1 cysteine within the ATP binding site (Figure 17).[106]



Hypothemycin
Residual activity at 0.2 µM
PKD1 = 34%
PKD2 = 31%

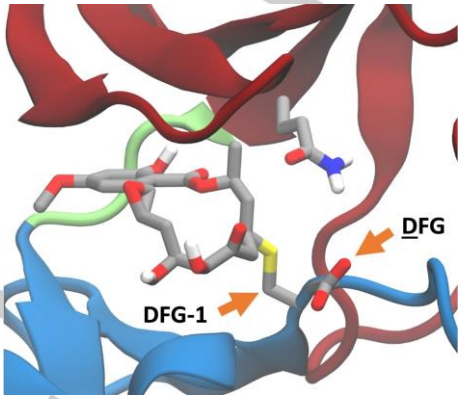


Figure 17. Top: structure and activity of covalent PKD inhibitor hypothemycin. Bottom: Presumed binding mode based on the crystal structure of ERK2 in complex with hypothemycin (PDB ID: 3C9W).

The aforementioned gradual implementation of kinome profiling studies has resulted in the availability of enormous amounts of activity data for known kinase inhibitors. Such profiling studies can be extremely valuable for studying polypharmacology or to set-up drug repurposing campaigns. Several noteworthy databases have been created and made publicly accessible providing a wealth of potent PKD inhibitors.[107-114] Table 2 displays a curated summary of these kinome screens with respect to the PKD family. This table clearly demonstrates that many kinase inhibitors have off-target activity to protein kinase D. A detailed overview of PKD inhibitors that have been identified in these large screenings is provided in the Supporting Information. Despite the public availability of this data, currently no repurposing studies have been performed on PKD.

3. Summary & Outlook

Several PKD inhibitors with diverse chemotypes have been reported throughout the past years. Traditionally, early-identified PKD inhibitors have mainly been used in biology to investigate PKD signaling in fundamental cellular processes. This wealth of information empowered the identification of protein kinase D as an attractive drug target for cardiac hypertrophy and several cancers. This has been the main incentive in the late 2000's to pursue potent, rationally designed PKD inhibitors. Despite that the use of structure-based approaches was hampered by the lack of any crystal structure of PKD's catalytic domain, still several potent inhibitors emerged from these drug discovery campaigns. Nonetheless, to date only few have reached preclinical stages mainly in the field of cancer treatment. Indeed, it currently seems that notwithstanding the accumulated evidence of cell signaling

experiments, PKD still needs more target validation for the treatment of cardiac hypertrophy. On the other hand, for cancer, the picture is clearly different. Multiple animal studies have demonstrated the potential of CRT0066101 to reduce the growth of pancreatic, colorectal and breast cancer in xenograft mice. Despite these remarkable results, the first PKD inhibitor yet remains to enter clinical trials.

One of the potential reasons for this hampered progression towards clinical trials is that many of the aforementioned inhibitors, including CRT0066101, still have a significant number of off-targets which could lead to unpredictable and undesired effects. In terms of kinome selectivity, the Novartis 3,5-diarylisoxazole compound series originally designed to treat cardiac hypertrophy, might be attractive to further validate in cancer treatment due to their apparent limited kinase off-targets. In addition, other opportunities to improve the kinome selectivity of ATP-directed PKD inhibitors reside in the development of irreversible inhibitors. Currently, no covalent inhibitors have been designed specifically for PKD. Protein kinase D harbors a cysteine residue in the ATP binding site at the DFG-1 position. This residue is present in only 10% of all known kinases thus offering opportunities to develop covalent inhibitors with PKD selectivity.[106, 115]

Table 2. Kinome profiling databases and the number of identified potent PKD inhibitors.[a]

Database	Number of compounds in database[b]	Number of identified inhibitors			Reference
		PKD1	PKD2	PKD3	
HMS LINCS Database[c]	182	38	27	34	[107]
MRC PPU Database[d]	254	51	-	-	[108]
Anastassiadis *et al.*, 2011[e]	178	15	16	21	[109]
Davis *et al.*, 2011[f]	72	11	9	15	[110]
Metz *et al.*, 2011[g]	3858	-	110	-	[111]
Published Kinase Inhibitor Set[h]	367	15	19	18	[112]
Klaeger *et al.*, 2017[i]	243	-	15	14	[113]
Published Kinase Inhibitor Set 2[j]	645	63	61	81	[114]

[a] Note that despite the similar assay set-ups, these biochemical results also rely on the applied ATP concentration. These potential differences were not considered in this analysis. [b] Number of compounds tested in total [c] IC_{50} cut-off ≤ 1 μM, RA cut-off 1 μM ≤ 50% and RA cut-off 10 μM ≤ 20% [d] RA cut-off 0.01 μM ≤ 70%, RA cut-off 0.1 μM and 1 μM ≤ 50% and RA cut-off 10 μM ≤ 20% [e] RA cut-off 0.5 μM ≤ 50% [f] K_i ≤ 1 μM [g] K_i < 1 μM [h] %inhibition cut-off at 1 μM ≥ 50% [i] EC_{50} cut-off ≤ 1 μM.

It is clear that future design and optimization of PKD inhibitors will face, next to kinome selectivity, another big challenge. The opposing roles of PKD's isoforms in cancer cell signaling have recently been subject of numerous studies and are gradually becoming understood. It appears that in general PKD1 has tumor suppressive functions while PKD2 and 3 are more frequently related to pro-oncogenic signaling. These biological findings are in stark contrast with the lack of progress in the development of isozyme specific PKD inhibitors. Being on the verge of understanding PKD's isozyme specific functions, much remains to be unraveled including the effects of selectively inhibiting specific PKD isoforms by means of small molecules in cancer cells. Such specific modulators would not only allow to selectively inhibit the tumor driving isoforms, but also leave potentially tumor protective isoforms untouched. Given the high sequence identity among the different PKD isoforms, such efforts would greatly benefit from structure-based approaches. However, even then the question remains to what extent this selectivity can be achieved within the catalytic site of protein kinase D. Therefore, allosteric inhibitors targeting less conserved regions of PKD could provide a breakthrough solution. Next to probing specific PKD isoforms, such inhibitors could also be less sensitive towards the elevated levels of ATP in cells. This typical activity drop is often encountered when progressing kinase inhibitors from pure enzymatic towards cellular environments. Finally, allosteric PKD inhibitors might also benefit from diminished kinome-wide off-target activity allowing for a better evaluation of the true biological responses triggered by small-molecule PKD inhibition.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Protein Kinase D • Cancer • Cardiac hypertrophy • CRT0066101 • 3-IN-PP1

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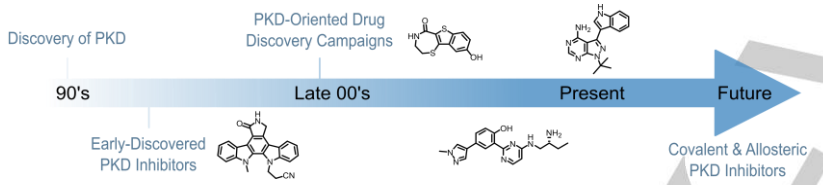

Protein kinase D (PKD) inhibitors: PKD is considered an attractive drug target for the treatment of cancer and cardiac hypertrophy. This review article provides an overview of the current landscape of PKD inhibitors. Next to a comprehensive discussion on biological properties, knowledge gaps are discussed delineating future research avenues.

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