

Project Title:

**IMAP-based Fluorescent Polarization Assay for HTS Protein
Kinase D Inhibitors**

Screening Center Name:

University of Pittsburgh Molecular Library Screening Center

Principal Investigator of Screening:

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Assay Submitter & Institution:

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Assay or Pathway Target:

Protein Kinase D

Probe PubChem Compound Identifier (CID):

PubChem-CID 755673 (PubChem-SID 4242787)



Specific Aim: Protein kinase D (PKD) is a novel family of serine/threonine kinases targeted by diacylglycerol. They regulate many fundamental cell functions including cell proliferation, survival, differentiation and protein trafficking, and have important roles in pathological conditions such as cardiac hypertrophy and cancer. Nonetheless, the mechanisms underlying PKD effects are not clearly understood and, thus, the role of PKD in cancer and other diseases has not been fully defined. This is partly due to the lack of effective pharmacological tools that specifically target PKD in normal and pathological processes. Therefore, we developed a high throughput screen (HTS)-ready, IMAP-based fluorescent polarization (FP) assay for the identification of PKD kinase inhibitors. The following specific aims were proposed to be carried out by the MSLCN via the Pittsburgh Molecular Library Screening Center:

- Aim 1.** To implement a validated IMAP-based PKD HTS primary assay to screen the full panel MSLCN compound library to identify small molecule inhibitors of PKD
- Aim 2.** To process primary hits through a secondary assay screening paradigm designed to: (a) evaluate the hit compounds for assay format interference; (b) confirm the hits and eliminate false positives using a 10-point concentration curve in the FP assay and a radiometric PKD kinase assay; (c) assess the specificity of the hits by conducting counter-screening using a HTS protein kinase C (PKC) kinase assay; (d) evaluate the hits for selectivity against different PKD isoforms and Ca²⁺/Calmodulin kinases.
- Aim 3.** To evaluate hit compounds and develop chemical probes for relevant biological systems, specifically (a) to test the hit compounds in a tertiary cell-based assay; (b) to design and develop novel chemical probes with improved selectivity and specificity.

Significance: This proposal is part of a global effort focused on investigating the critical roles of PKD, the novel diacylglycerol/phorbol ester target, in normal cell functions and in pathogenic processes. The goal of this proposal is to use HTS to discover and then develop novel potent and selective small molecular inhibitors of PKD. The significance of developing potent and selective PKD inhibitors are several folds: 1) as probe for determining the biological functions of PKD and for dissecting the signaling events regulated by PKD *in vivo* and *in vitro*; 2) as potential drugs that could be applied clinically or as agents on which new drug designs could be based; 3) as a novel class of drugs/inhibitors that target the PKC signaling. Since PKD is downstream of PKC and the substrate specificity of PKD is much more restricted, inhibitors for PKD will likely have more selectivity and less toxicity compared to those for PKC. Preliminary data strongly support the feasibility of an IMAP-based fluorescent polarization assay for high throughput screen of PKD inhibitors. Strong likelihood for success exists, as a preliminary small-scale screening has generated meaningful hits. Specific inhibitors for PKD have not been documented. The success of this proposal will fill this gap that has impeded the progress of research in the field for over a decade, and most importantly, it will open opportunities for research in whole animals and facilitate transfer of basic research discovery to clinical settings. Overall, this proposal represents a major drug discovery effort towards an emerging critical biological target of cancer and heart disease. Ultimately PKD-targeted drugs could provided novel treatment strategies for many disease caused by aberrant DAG signaling.

Rationale: Despite the wealth of evidence for the divergent biological functions of PKD and their clinical relevance, there are no inhibitors that specifically target PKD thus far. The most frequently used PKD inhibitor Gö6976, which inhibits PKD at IC₅₀ of 20 nM, is in fact a indolocarbazole class of PKC inhibitor that inhibits cPKC isoforms at higher potency (IC₅₀ is 2-6 nM for PKC α and PKC β I (37), and also inhibit phosphorylase kinase, checkpoint kinase, and mitogen- and stress-activated kinase 1 (37-39). Its value for delineating PKD signaling pathways or for clinical application is questionable because of the apparent lack of specificity. Another compound resveratrol (trans-3,4',5-trihydroxystilbene), an antioxidant and effective chemopreventive agent, is reported to inhibit PKD at IC₅₀ 200 μ M *in vitro* and 800 μ M *in vivo*. These IC₅₀ values greatly exceed the dose of resveratrol that produces other cellular effects (e.g., inhibition of cyclooxygenase), which limits its *in vivo* application (40). Thus, potent and selective inhibitors of PKD are clearly needed to further clarify the contribution of PKD in biological processes and the underlying molecular mechanisms, and to facilitate long-term therapeutic development and applications. The discovery of novel small molecule inhibitors of PKD will provide the necessary tools to carry out these studies.



Assay Implementation and Screening

Pubchem Bioassay names

1. Primary Screen: Fluorescence polarization assay for PKD inhibitors (**AID 797**)
2. Secondary Screens: See Appendices for specific assays performed (**AID** not presently available).

Primary Assay Description as defined in Pubchem: Protein kinase D (PKD) is a novel family of serine/threonine kinases targeted by diacylglycerol. They regulate many fundamental cell functions including cell proliferation, survival, differentiation, and protein trafficking, and have important conditions such as cardiac hypertrophy and cancer. Unfortunately, the mechanisms underlying these effects of PKD are not clearly understood and the role of PKD in cancer and other diseases has not been fully defined. This is partly due to the lack of effective pharmacological tools that specifically target PKD in normal cellular processes and in pathological conditions. The immediate goal of this proposal is to demonstrate the feasibility of an IMAP-based fluorescent polarization (FP) assay for high throughput (HTS) of PKD inhibitors. The assay we proposed is a 384-well, small volume format that has been adapted for high throughput screening. The objective is to discover novel potent and selective small molecule inhibitors of PKD that will be helpful in understanding the biological relevance of PKD and have the potential for long-term therapeutic application.

PKD HTS Assay Conditions:

- (1) Add 2 μ L of a 3X substrate peptide and ATP (300 nM/60 μ M) mixture to appropriate wells.
- (2) Add 2 μ L of a 30 μ M concentration of test compound or controls (3X) to appropriate wells.
- (3) Add 2 μ L of 0.18 Units/mL PKD enzyme (3X) to each well.
- (4) Incubate for 1.5 hours at room temperature.
- (5) Add 18 μ L of Binding reagent to each well and incubate for 2 hours.
- (6) Collect data at A_{485}/A_{525} .

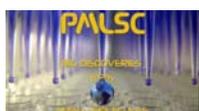
Center Summary of Primary and Secondary Screening Assays

- Primary HTS – A total of 196,173 compounds were screened at 10 μ M (**AID 797**)
 - 109 (0.06%) compounds exhibited $\geq 50\%$ inhibition
 - Compounds ($\geq 50\%$ inhibition) were provided by Biofocus DPI via two cherry pick orders
 - Re-test of primary HTS actives to confirm their activity in the IMAP-based PKD assay
 - 16 compounds confirmed as PKD inhibitors exhibiting $\geq 50\%$ inhibition
 - Test of the primary HTS actives to determine if they interfered with the assay format
 - 5 compounds interfered with the assay format
- 10-point concentration response confirmation of PKD inhibitory activity
 - 1 compound (11.1%) exhibited $IC_{50} < 1$; 2 compounds (22.2%) exhibited $1 \leq IC_{50} \leq 5$; 5 compounds (55.5%) exhibited $5 \leq IC_{50} \leq 10$; and 1 compound (11.1%) exhibited $IC_{50} > 10$.
 - 10-point concentration response of confirmed actives in PLK1, CDK7 and AKT IMAP-based kinase assay
 - 9 confirmed actives were screened in IMAP-based PLK1, CDK7 and/or AKT assay to ascertain the specificity of the PKD inhibitory activity (see Appendices for data summary)

Secondary Screening Assays Performed by the Assay Provider

Radiometric kinase assay for confirmation of compound inhibitory activity

1. Assay protocol. The radiometric kinase assay was carried out by co-incubating 50 ng recombinant human PKD/PKC μ with 20 μ M ATP, 2.5 μ g syntide-2 (a PKD substrate peptide), and 0.5 μ Ci of [γ - 32 P]ATP in a final volume of 50 μ L. The reaction was allowed to proceed at 30°C for 7 min. An aliquot of the reaction mixture was then spotted on p81 paper, washed in 5% phosphoric acid, and counted in a liquid scintillation counter.
2. Results. Four (out of nine) compounds have to assayed to date in the radiometric kinase assay using a 10-point concentration dose range (of each compound of interest). Of the four compounds examined, one



compound exhibits an $IC_{50} \leq 500$ nM, one compound exhibits an $500 \text{ nM} \leq IC_{50} \leq 1 \mu\text{M}$ and two compounds exhibit an $1 \mu\text{M} \leq IC_{50} \leq 5 \mu\text{M}$. Data collected to date is summarized in the Appendices.

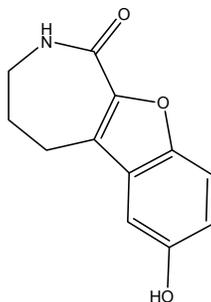
Probe Optimization

Chemical name of probe compound: 7-hydroxy-2,3,4,5-tetrahydro-[1]benzoxolo[2,3-c]azepin-1-one

Molecular formula: $C_{12}H_{11}NO_3$

MW: 217.22064 g/mol

Draw probe chemical structure and show stereochemistry if known:



Pubchem CID 755673

Pubchem SID 4242787

Average $IC_{50} = 264 \pm 27$ nM

Describe mode of action for biological activity of probe

No mechanism of action/enzyme kinetics data is presently available.

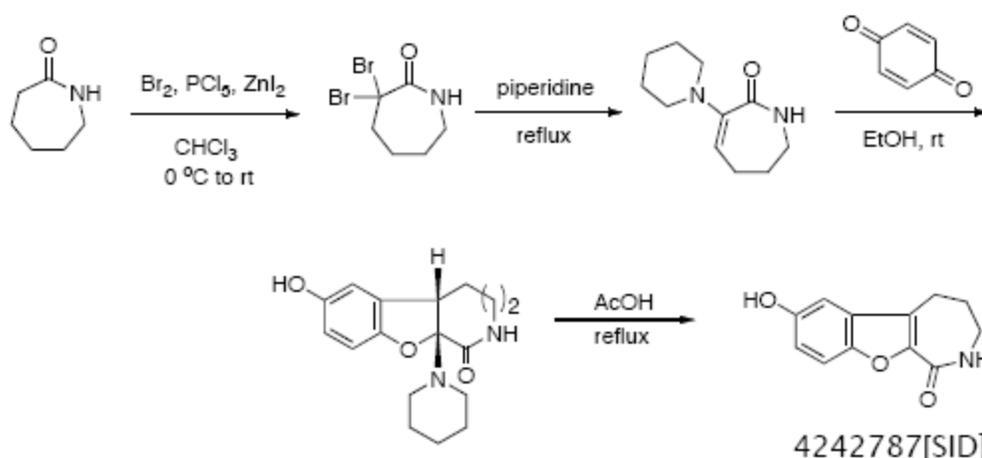
Has the compound been provided to the MLSMR?

A sample of this compound is present in the MLSMR.

Center comments on chemistry strategy leading to probe identification: This compound was identified after the primary screen of the full panel PMLSC library and therefore, is a constituent of the NIH MLSMR. There are a number of similar structures (analogues) of this compound within the library and these compounds provided a preliminary SAR.

Detailed synthetic pathway for making the probe:





Center Summary of Probe Properties

Pubchem Listed Properties:

Molecular Weight: 217.22064 g/mol, Molecular Formula: C₁₂H₁₁NO₃, Hydrogen Bond Donor Count: 2, Hydrogen Bond Acceptor Count: 3, Rotatable Bond Count: 0, Tautomer Count: 6, Exact Mass: 217.073893, Monoisotopic Mass: 217.073893, Topological Polar Surface Area: 62.5, Heavy Atom Count: 16, Charge: 0, Complexity: 294, Isotope Atom Count: 0, Defined Atom StereoCenter Count: 0, Undefined Atom StereoCenter Count: 0, Defined Bond StereoCenter Count: 0, Undefined Bond StereoCenter Count: 0, Covalently-Bonded Unit Count: 1

Appendices

Secondary screens performed on the confirmed PKD actives

1. AKT IMAP fluorescence polarization – determine if compounds inhibit AKT activity (in the same assay format)
2. CDK7 IMAP Time-resolved fluorescence energy transfer – determine if compounds inhibit CDK7 activity
3. PLK1 IMAP Time-resolved fluorescence energy transfer – determine if compounds inhibit PLK1 activity
4. Effects of DTT on the inhibitory effects of confirmed actives
5. Determinations of peroxide generation (of the confirmed actives)

Specificity data of the first eight confirmed PKD actives (from IMAP-based assay formats)

Compound Pubchem SID	PKD IC ₅₀ (μM)	CDK7 IC ₅₀ (μM)	PLK1 IC ₅₀ (μM)	AKT IC ₅₀ (μM)	DTT (25 mM) IC ₅₀ (μM)
4242787	0.625	31.4	16.7	>50	0.383
17407044	8.8	>50	0.410	>50	3.6
858234	4.3	>50	>50	>50	3.6
14741443	6.0	>50	>50	>50	2.4
17510359	6.7	6.2	>50	>50	3.1
14740154	16.5	>50	>50	>50	10.6
855999	5.9	12.4	6.6	>50	3.7
14719315	1.3	8.4	12.1	>15	0.8

Radiometric kinase assay data collected to date

Compound SID	EXPT 1 IC ₅₀ (nM)	EXPT 2 IC ₅₀ (nM)	EXPT 3 IC ₅₀ (nM)	Average IC ₅₀ ± SD (nM)
14719315	557.7	756.2	730.8	671.9 ± 69
4242787	208.8	291.9	290.7	263.8 ± 27.5
855999	1295	1131	10863	1170 ± 63.5
858234	1381	1227	1232	1280 ± 50.5

The remaining 5 compounds are presently being assayed in the radiometric PKD kinase assay.

Cross Target Query on Pubchem Database

Pubchem SID	Pubchem CID	# Assays	Active flags	Primary HTS Actives	Dose Response Actives
4242787	755673	210	3	3	0

