Project Title:

High throughput screening assay for inhibitors of the Polo box domain of Human Polo-like kinase 1

Grant Number:

MH078944

Screening Center Name:

University of Pittsburgh Molecular Screening Center

Principal Investigator of Screening Center:

John S. Lazo

Assay Submitter & Institution:

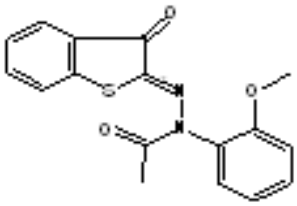
Michael B. Yaffe
Massachusetts Institute of Technology

Assay or Pathway Target:

Polo box domain of Human Polo-like kinase 1

Probe PubChem Compound Identifier (CID):

PubChem-CID 5401876 (PubChem-SID 47212999)



PubChem-SID 47212999,
PubChem-CID 5401876
Plk-1-PBD Binding IC_{50} 13.28 μM

Specific Aim: The specific aim of this project is to identify lead compounds for anti-cancer drug development using a fluorescence-based high-throughput screen for inhibitors of Polo-like kinase 1 (Plk1).
Aim 1. Screen MLSCN compound libraries for inhibitors of the polo box domain of polo-like kinase-1 (Plk-1-PBD) using a high throughput-ready fluorescence polarization (FP) assay.
Aim 2. Follow up primary screen of PBD inhibitors with secondary biochemical and cell based assays to confirm hits.

Significance: The Polo-like kinases (Plks) play important roles in many cell cycle-related events including the initiation of mitosis, chromosome segregation, centrosome maturation, bipolar spindle formation, regulation of anaphase-promoting complex and execution of cytokinesis [1-6]. The prototypic Polo kinase was originally identified in flies, in which mutants resulted in abnormal spindle poles [11, 12]. A single Polo family member is found in flies, budding yeast, and fission yeast (Polo, CDC5 and Plo1, respectively) [11, 13, 14], while 3 Plk family members are found in humans, mice and frogs (Plk1/Plx1 [15, 16], Plk2/Snk/Plx2 [17, 18] and Plk3/Fnk/Prk/Plx3 [17, 19, 20], respectively). One human family member, Plk1, as well as its homologues in Xenopus (Plx1) and budding yeast (Cdc5) plays a particularly important role in coordinating multiple events during mitosis. Plk1/Plx1/Cdc5 is critical for proper centrosome maturation and spindle assembly. Plk1/Plx1/Cdc5 protein expression peaks during late G2 and M phases, where it localizes to centrosomes and spindle pole bodies during prophase and metaphase. During this time, Plk1/Plx1 likely phosphorylates a number of key centrosome-associated proteins [7, 21-26], including the mitotic phosphatase Cdc25C to initiate the Cdc25C-Cdc2 amplification loop at the onset of M phase [23, 27-32]. At metaphase, Plk1 activates the anaphase-promoting complex (APC) to upregulate the ubiquitin-dependent proteolytic degradation of factors that regulate passage through mitosis. In addition, Plk1 and its homologues play important roles in the later stages of mitosis, particularly in anaphase and cytokinesis. In budding yeast, for example, Cdc5 phosphorylates the Scc1 subunit of the cohesin complex, which holds sister chromatids together until the start of anaphase [33]. Phosphorylation of Scc1 by Cdc5 facilitates proteolytic cleavage of Scc1 by separase to allow sister chromatid separation upon anaphase onset. Plk1 re-localizes to the spindle midzone during late anaphase, possibly playing roles in regulating components of microtubules and kinetochores, and subsequently re-localizes to the mid-body during telophase and cytokinesis, where it appears to play a critical role in formation of the contractile ring at the site of cytoplasmic division [5, 6, 16, 22, 29, 34].

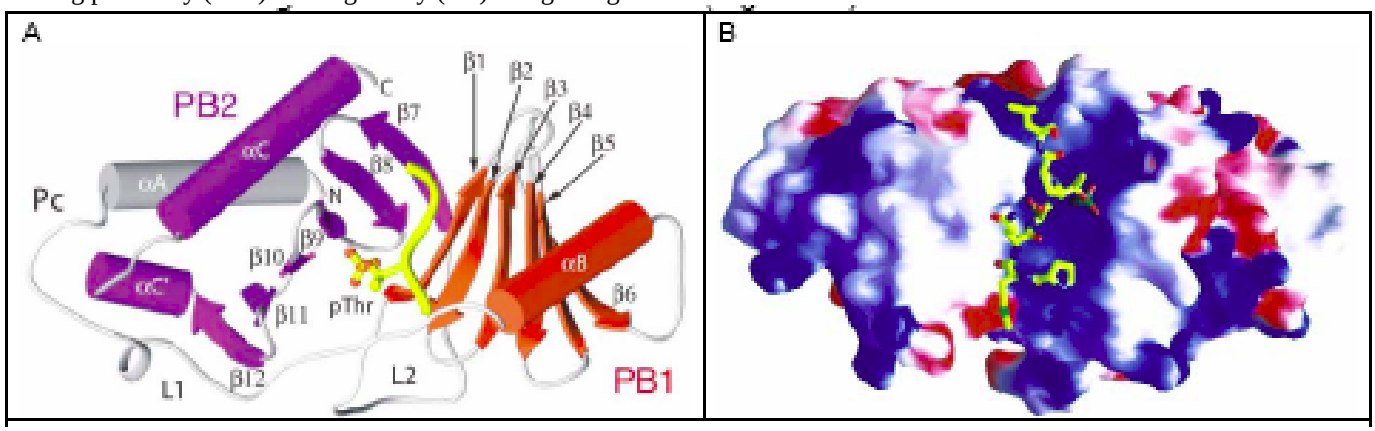
Plk1 is highly expressed in proliferating cells and in various human tumors including squamous cell cancers of the head and neck, melanomas, gliomas, esophageal, colorectal and lung carcinomas and endometrial, ovarian and breast cancers [35-46]. High levels of Plk1 expression in tumors often correlate with poor prognosis. Constitutive expression of Plk1 in NIH 3T3 cells causes malignant transformation and induces tumor growth in nude mice [47]. Importantly, antisense oligonucleotides that down-regulate Plk1 cause decreased cell proliferation in MDA-MB-435 breast cancer cells and A549 non-small cell lung cancer cells in vitro and in tumor regression when these cells were xenografted onto nude mice [48, 49]. Interestingly the viability of non-transformed cells such as amniocytes, mesangial cells and skin fibroblasts was unaffected by these types of Plk1 down-regulation. These observations strongly indicate that Plk1 is likely to be a highly effective target for anti-cancer drug design.

Rationale: Exploiting the phosphopeptide sequence specificity of the Polo box domain for Plk1 inhibitor screening. The phosphoserine/phosphothreonine-based sequence motif recognized by the Plk1 PBD shows partial overlap with the phosphorylation sequence generated by mitotic Pro-directed kinases such as cyclin-Cdks. This observation suggests that phosphorylation by Cdk kinase activity may prime certain substrates for binding to, and subsequent phosphorylation by, the kinase domain of Plk1. The PBD, for example, is required for the localization of Plk1 to centrosomes during prophase, where at least two known Cdk and Plk1 substrates are found: CyclinB and Cdc25C [50-52]. Inhibiting the normal function of the PBD profoundly disrupts Plk1localization, Cdc25C phosphorylation, and cell division. We showed previously that the

centrosomal localization of the PBD could be blocked by an optimal phosphopeptide, but not by the corresponding unphosphorylated peptide [9]. Furthermore, overexpression of a dominant negative wild-type Plk1 PBD, but not a PBD mutant that was unable to bind phosphopeptides caused M-phase arrest and aneuploidy in HeLa cells, by competing with endogenous Plk1 for centrosome binding [10]. These results strongly imply that inhibitors that block phosphopeptide binding by the PBD will impede the interaction of Plk1 with its substrates, disrupt its normal subcellular localization, and arrest cell division.

Using an immobilized library of partially degenerate phosphopeptides to screen cDNA library for novel phosphopeptide binding domains, the Yaffe laboratory previously identified the Plk1 Polo box domain (PBD) as a novel pSer/pThr-binding domain [9]. They determined the optimal binding motif of the PBD to be Ser-[pSer/pThr]-Pro/X, using multiple pThr- and pSer-containing peptide libraries and subsequently characterized the binding of the PBD to an optimal phosphopeptide ligand with the core sequence Met-Gln-Ser-pThr-Pro-Leu. Isothermal titration calorimetry showed that this optimal phosphopeptide bound to the Plk1 PBD with a 1:1 stoichiometry and a dissociation constant of 280 nM. These findings provided an optimal substrate peptide to be used in small molecule competition assays to identify lead compounds that disrupt Plk1 PBD:phosphopeptide interaction. The Polo box domain (PBD) contains two ~70 amino acid sequence motifs (Polo boxes) that are highly conserved in all Polo-like kinases [8, 53, 54]. The Yaffe group have produced large amounts of recombinant Plk1 PBD and solved the high resolution X-ray crystal structure of the PBD in complex with its optimal phosphopeptide [10]. Each of the Polo boxes consisted of β6α structures that together formed a 12-stranded β sandwich domain (Figure 1A).The phosphopeptide binding site was located in a conserved positively charged cleft located at the edge of the Polo-box interface (Figure 1B).

Figure 1. Crystal structure of the Polo box domain in complex with an optimal phosphopeptide. A. Ribbon figure highlighting the Polo boxes (PB1 and PB2). The bound phosphopeptide is shown in yellow. B. Molecular surface representation showing positively (blue) and negatively (red) charged regions.



A fluorescence polarization (FP) assay was developed for HTS using a fluorescently labeled peptide ligand based on the previously determined optimal phosphopeptide. The FITC phospho-peptide consisted of the sequence Met-Gln-Ser-pThr-Pro-Leu-Lys and included a fluorescein isothiocyanate (FITC) fluorophore conjugated to the ε-amino group of the C-terminal Lys. Upon binding to the PBD protein, the FITC-conjugated phospho-peptide shows an increased FP signal. This increase in FP is lost if binding is inhibited by competition with unlabeled phospho-peptide or by small molecule compounds. A His6-GST-tagged construct of the PBD was used for the FP assays. The large native molecular mass (120 kDa) of this dimeric fusion construct was expected to provide a greater increase in FP signal upon binding to FITC-phospho-peptide relative to a 35 kDa untagged monomeric PBD construct. The His6-GST-PBD was overexpressed in Escherichia coli and purified by cation exchange, Ni-metal affinity and gel filtration chromatography, with final yields of at least ~4-5 mg /

liter of culture. The FP assay consists of the addition of His6-GST-PBD protein to wells of 384-well plates followed by the addition of compounds (DMSO stocks) and finally the addition of FITC-labeled phospho-peptide.

Assay Implementation and Screening

PubChem Bioassay Names:

1. Primary Screen: High throughput fluorescence polarization-based assay to screen for small molecule inhibitors of the Polo box domain (PBD) of Plk1. **AID 693**
2. Concentration Response Confirmation Assay: Concentration Response fluorescence polarization-based assay to confirm small molecule inhibitors identified in the Polo box domain (PBD) of Plk1 Primary HTS. **AID 877**
3. Secondary Assays: Concentration Response Redox Cycling H2O2 Generation assays to characterize small molecule inhibitors identified in the Polo box domain (PBD) of Plk1 Primary HTS. 0.5 mM DTT **AID 876** and 1.0 mM DTT **AID 936.**
4. Chemistry Follow Up: Concentration Response fluorescence polarization-based assay to test PMLSC Chemistry Core synthesized analogs of 861574, a confirmed hit from the Polo box domain (PBD) of Plk1 Primary HTS. **AID 1082.**
5. Chemistry Follow Up II: Concentration Response fluorescence polarization-based assay to test PMLSC Chemistry Core synthesized analogs of confirmed hits from the Polo box domain (PBD) of Plk1 Primary HTS. **AID 1057.**
6. Purchased Analogs Follow Up: High throughput fluorescence polarization-based assay to screen for small molecule inhibitors of the Polo box domain (PBD) of Plk1 to follow up on purchased Analogs of Selected Hits from the screen. **AID 1053.**
7. Concentration Response fluorescence polarization-based assay to test purchased Analogs of Selected Hits from the Polo box domain (PBD) of Plk1 Primary HTS. **AID 1083.**

Primary Assay Description as defined in PubChem:

The Polo-like kinases (Plks) play important roles in many cell cycle-related events including the initiation of mitosis, chromosome segregation, centrosome maturation, bipolar spindle formation, regulation of anaphase-promoting complex and execution of cytokinesis. The prototypic Polo kinase was originally identified in flies, in which mutants resulted in abnormal spindle poles. A single Polo family member is found in flies, budding yeast, and fission yeast (Polo, CDC5 and Plo1, respectively), while 3 Plk family members are found in humans, mice and frogs (Plk1/Plx1, Plk2/Snk/Plx2 and Plk3/Fnk/Prk/Plx3 respectively). One human family member, Plk1, as well as its homologues in Xenopus (Plx1) and budding yeast (Cdc5) plays a particularly important role in coordinating multiple events during mitosis. Plk1/Plx1/Cdc5 is critical for proper centrosome maturation and spindle assembly. Plk1/Plx1/Cdc5 protein expression peaks during late G2 and M phases, where it localizes to centrosomes and spindle pole bodies during prophase and metaphase. During this time, Plk1/Plx1 likely phosphorylates a number of key centrosome-associated proteins, including the mitotic phosphatase Cdc25C to initiate the Cdc25C-Cdc2 amplification loop at the onset of M phase. At metaphase, Plk1 activates the anaphase-promoting complex (APC) to upregulate the ubiquitin-dependent proteolytic degradation of factors that regulate passage through mitosis. In addition, Plk1 and its homologues play important roles in the later stages of mitosis, particularly in anaphase and cytokinesis. In budding yeast, for example, Cdc5 phosphorylates the Scc1 subunit of the cohesin complex, which holds sister chromatids together until the start of anaphase. Phosphorylation of Scc1 by Cdc5 facilitates proteolytic cleavage of Scc1 by separase to allow sister chromatid separation upon anaphase onset. Plk1 re-localizes to the spindle midzone during late anaphase, possibly playing roles in regulating components of microtubules and kinetochores, and

subsequently re-localizes to the mid-body during telophase and cytokinesis, where it appears to play a critical role in formation of the contractile ring at the site of cytoplasmic division.

Plk1 is highly expressed in proliferating cells and in various human tumors including squamous cell cancers of the head and neck, melanomas, gliomas, esophageal, colorectal and lung carcinomas and endometrial, ovarian and breast cancers. High levels of Plk1 expression in tumors often correlate with poor prognosis. Constitutive expression of Plk1 in NIH 3T3 cells causes malignant transformation and induces tumor growth in nude mice. Importantly, antisense oligonucleotides that down-regulate Plk1 cause decreased cell proliferation in MDA-MB-435 breast cancer cells and A549 non-small cell lung cancer cells in vitro and in tumor regression when these cells were xenografted onto nude mice. Interestingly the viability of non-transformed cells such as amniocytes, mesangial cells and skin fibroblasts was unaffected by these types of Plk1 down-regulation. These observations strongly indicate that Plk1 is likely to be a highly effective target for anti-cancer drug design.

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Using an immobilized library of partially degenerate phospho-peptides to screen cDNA library for novel phospho-peptide binding domains, our laboratory previously identified the Plk1 Polo box domain (PBD) as a novel pSer/pThr-binding domain. We determined the optimal binding motif of the PBD to be Ser-[pSer/pThr]-Pro/X, using multiple pThr- and pSer-containing peptide libraries and subsequently characterized the binding of the PBD to an optimal phospho-peptide ligand with the core sequence Met-Gln-Ser-pThr-Pro-Leu. Isothermal titration calorimetry showed that this optimal phospho-peptide bound to the Plk1 PBD with a 1:1 stoichiometry and a dissociation constant of 280 nM. These findings have provided us with an optimal substrate peptide to use in small molecule competition assays to identify lead compounds that disrupt Plk1 PBD:phospho-peptide interaction.

A fluorescence polarization (FP) assay was developed using a fluorescently labeled peptide ligand based on the previously determined optimal phospho-peptide and transferred to the PMLSC. The FITC phospho-peptide consisted of the sequence Met-Gln-Ser-pThr-Pro-Leu-Lys and included a fluorescein isothiocyanate (FITC) fluorophore conjugated to the ε-amino group of the C-terminal Lys. Upon binding to the PBD protein, the FITC-conjugated phospho-peptide shows an increased FP signal. This increase in FP is lost if binding is inhibited by competition with unlabeled phospho-peptide or by small molecule compounds. A His6-GST-tagged construct of the PBD was used for the FP assays. The large native molecular mass (120 kDa) of this dimeric fusion construct was expected to provide a greater increase in FP signal upon binding to FITC-phospho-peptide relative to a 35 kDa untagged monomeric PBD construct. The His6-GST-PBD was overexpressed in Escherichia coli and purified by cation exchange, Ni-metal affinity and gel filtration chromatography, with final yields of at least ~4-5 mg / liter of culture. Purified protein could be conveniently stored in frozen aliquots at -

80ºC. The FP assay consists of the addition of His6-GST-PBD protein to wells of 384-well plates followed by the addition of compounds (DMSO stocks) and finally the addition of FITC-labeled phospho-peptide.

Plk-1-PBD HTS Fluorescence Polarization Binding Assay Conditions:

Reagents & Materials:
The His6-GST-PBD protein, FITC-labeled phospho-peptide and unlabeled phospho-peptide were provided to the PMLSC by the assay provider (Dr. Michael Yaffe, MIT).

10X Assay Buffer: 500 mM Tris, 2 M NaCl, 20 mM DTT , 0.05 % Tween 20, pH 8.0.

Tris HCl	60.57 g
NaCl	116.88 g
Tween 20	500 uL (0.05%)
dH2O	800mL

pH to 8.0 with NaOH
add dH2O to 1000mL

DTT: 200 mM Stock in -20 ◦C.

His6-GST-PBD protein: 150 ng in 5 uL of 3X assay buffer. 50 uL aliquot of His–GST-PBD @ 70 mg/mL provided by the assay provider was diluted to 10 mg/mL by adding 300 uL of 1X assay buffer; 50 mM Tris, 200 mM NaCl, 2.0 mM DTT , 0.005 % Tween 20, pH 8.0. The protein was then aliquoted and stored at – 80 °C. To prepare 150 ng His–GST-PBD in 5 uL of 3X assay buffer add: 160 uL of 10 mg/mL His–GST-PBD + 16.0 mL of 10X assay buffer, + 1.6 mL of 200 mM DTT + 35 mL of H2O.

FITC-Phospho-peptide : 30 uM stock of FITC-Phospho-peptide in 10 mM Tris pH 8.0 + 1mg/mL BSA. Add 1.3 mg FITC-phospho-peptide to 33.53 mL of Tris HCl pH 8.0 and sonicate in water bath ~ 5 min to make a 30 uM stock of FITC-Phospho-peptide, aliquot and store at – 80 °C. Immediately prior to the assay dilute the 30 uM FITC-phospho-peptide to 30 nM in 10 mM Tris HCl pH 8 + 1.0 mg/mL BSA. Transfer 5 uL to each well of a 384-well of the assay plate ~ 10 nM FITC-phospho-peptide final.

Unlabeled-Phospho-peptide: 300 uM stock in 10 mM Tris HCl pH 8.0. Add 3.7 mg of unlabeled phospho-peptide to 13.2 mL of 10 mM Tris HCl pH 8.0, sonicate for 10 minutes, aliquot and store at – 80 °C. Immediately prior to the assay dilute the 300 uM unlabeled phospho-peptide to 30 uM in 10 mM Tris HCl pH 8.0 and transfer 5 uL per well for a final concentration of 10 uM.

Compounds: 5.0 uL/well ~ 20 µM final (2% DMSO). Dilute compounds in de-ionized H2O to 60 uM, 6% DMSO and transfer 5 uL per well to give a final assay volume of 15 uL and 20 uM compound in 2% DMSO.

Maximum Control: 5.0 uL/well of 6% DMSO in Tris HCl pH-8.0.

Minimum Control: 5.0 uL/well of 30 uM unlabeled phospho-peptide in 6% DMSO in Tris HCl pH 8.0.

50 % Control: 5.0 uL/well of 1 uM unlabeled phospho-peptide in 6% DMSO in Tris HCl pH 8.0.

PLK1-PBD Binding Assay Protocol:

1. Transfer 5 uL of diluted compounds and plate controls to wells of 384-well black low-volume 384-well assay plate, Greiner # 784076.
2. Transfer 5 uL/well of 3x PLK1-PBD enzyme solution to assay plate (150 ng/well final).
3. Centrifuge plate at 500 r.p.m. 1 min.
4. Transfer 5 uL/well of 30 nM FITC-phospho-peptide in 10 mM Tris HCl pH 8.0 + 1.0 mg/ml BSA solution to assay plate (10 nM FITC-phospho-peptide final).
5. Centrifuge plate at 500 r.p.m. 1 min.
6. Incubate assay plate at ambient temperature for 45 min.
7. Read fluorescence polarization (mP units) at Ex 488 nm Em 520 nm on the M5 plate reader.

The PLK-1PBD binding assay conducted at the PMLSC utilized a Z score statistical scoring method to identify active compounds (Brideau et al, 2003 J. Biomolecular Screening 8(6): p634-637. The Z score for a compound is computed on a plate-by-plate basis. The Z score for the raw fluorescent intensity value Xi is defined as Zi = (Xi-Xm)/Sm, where Xm is the mean of all the raw fluorescent intensity values of the compounds on a plate (n=320), and Sm is the standard deviation of all these values. A cut off Z score of -3 was selected as the active criterion for the PLK-1-PBD binding HTS.

PLK-1-PBD binding HTS Activity scoring rules:

PUBCHEM_ACTIVITY_OUTCOME
1 - Substance is considered inactive when Z-score is > -3
2 - Substance is considered active when Z-score is </= -3
3 - Substance activity outcome is inconclusive

PUBCHEM_ACTIVITY_SCORE
0-40 scoring range is reserved for primary HTS data
a) if Z-score is </= -3, the score is 40
b) if Z-score is > -3 the score is 10

Center Summary of the Primary Screening Assays:

- A total of 97090 compounds were screened at 20 μM in the primary screen, **AID 693.**
  - 127 (0.13%) compounds met the active criterion of Z-score </= -3
  - Leadscope classification & clustering analysis of 113 active structures in the database
    - 66 singletons and 21 clusters of 3 or 2 related compounds
- 127 compound cherry pick order submitted, 117 compounds available from Biofocus-DPI were tested in 10-point concentration response assay, **AID 877.**
  - 15/117 (12.8%) were confirmed with IC50s < 50 μM
  - Leadscope classification & clustering
    - 6 singletons & 4 clusters

Center Summary of the Hit Characterization Assays:

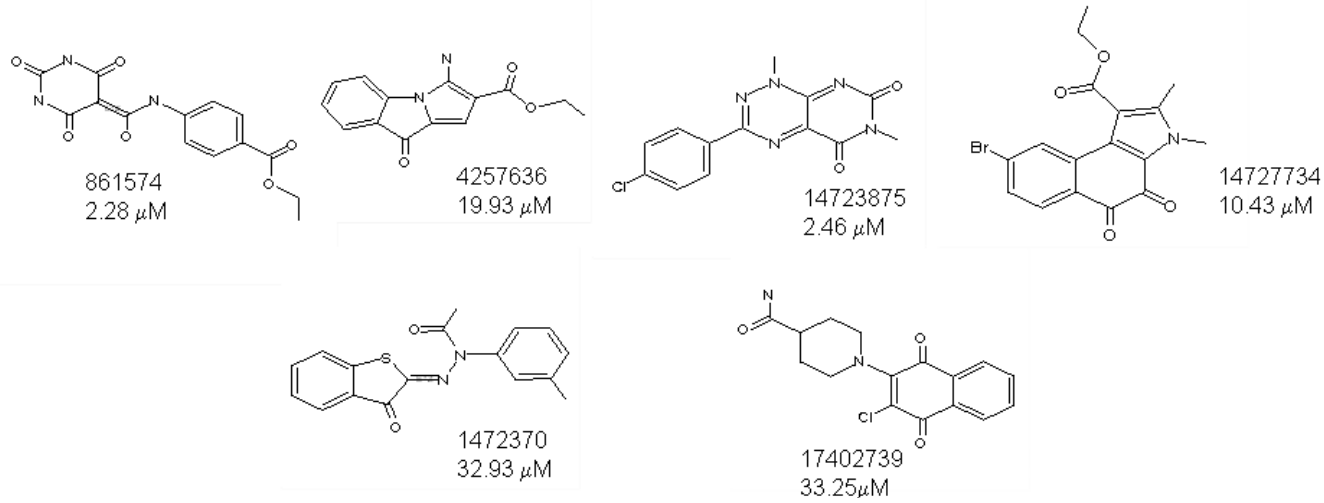
- Compound and DTT dependent Redox Cycling Generation of H_2O_2
  - 117 compounds tested in 10-point, 2-fold dilution series, concentration dependence assays starting at 50 μM

- 0.5 mM DTT ~ 6 of 117 exhibit H_2O_2 generation, **AID 876.**
- 1.0 mM DTT ~ 6 of 117 exhibit H_2O_2 generation, **AID 936.**

Compounds that were found to be capable of redox cycling in the presence of DTT to generate significant levels of H_2O_2 were considered to be likely false positives acting through an indirect effect of H_2O_2 on cysteine residues in the active site or contributing to the secondary/tertiary structure of proteins. Such compounds exhibit promiscuous bioactivity profiles in PubChem especially with regard to Protein Tyrosine Phosphatase and cysteine protease targets. Of the six compounds considered suitable for follow up (Fig 2), only PubChem-SID 14727734 was found to be capable of redox cycling in the presence of DTT to generate H_2O_2.

Figure 2. PLK-1-PBD Binding Inhibitors Selected for Follow Up.

	PLK-1-PBD Binding Assay				Redox Cycling Assay 0.5 mM DTT				Redox Cycling Assay 1.0 mM DTT			
Pubchem SID	% INHIB @ 50 uM	IC50 uM	R^2	HILL_SLOPE	% ACT @ 50 uM	EC50	R^2	HILL_SLOPE	% ACT at 50 uM	IC50	R^2	HILL_SLOPE
861574	108.59	2.277	0.948	0.970	-3.474	>50	0.295	-65.300	-2.243	>50	0.478	-10.315
4257636	75.68	19.928	0.828	0.687	-3.924	>50	0.894	-45.278	-2.901	>50	0.656	-22.669
14723875	78.54	2.460	0.835	1.485	-5.791	>50	0.563	-57.005	-3.575	>50	0.667	-10.275
14727734	73.55	10.435	0.929	1.592	132.712	0.614	0.941	164.095	59.461	0.723	0.889	26.892
14742370	79.42	32.934	0.919	2.980	-3.059	>50	0.148	-73.468	-1.942	>50	0.236	-26.613
17402739	66.30	33.256	0.829	1.073	-5.717	>50	0.743	-5.872	-4.785	>50	0.436	-2.678



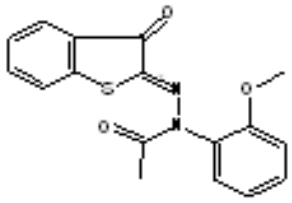
Summary of Secondary Assays Performed by Assay Provider:

The Yaffe group tried some co-crystallization trials with the Plk-1-PBD hit compound PubChem-SID 861574, which produced crystals, but revealed no electron density corresponding to the 861574 compound. The PMLSC chemistry core selected 7 analogs related to 861574 from the MLSMR and synthesized an additional 30 analogs of 861574, but unfortunately none of these analogs exhibited activity in the Plk-1-PBD binding assay, AID 1082. The MLSMR cherry picked sample of 861574 was consistently active in the Plk-1-PBD binding assay, but the LC-MS analysis indicated that the cherry picked sample supplied to the PMLSC was significantly different to the LC-MS analysis of the sample when it was first placed in the collection, indicating that the sample had changed significantly with storage in DMSO. Two purchased samples of 861574 from commercial vendors failed to exhibit activity in the Plk-1-PBD binding assay when first tested, but when stored for 1-2 weeks in DMSO at ambient temperature or at 37 ºC, the samples changed color and acquired

significant levels of activity in the Plk-1-PBD binding assay, consistent with the MLSMR cherry picked sample. The 861574 sample "aged" in DMSO has been fractionated by HPLC and tested in the bioassay. Efforts are underway to identify the structure(s) of the active component(s) in the active fractions.

Probe Optimization:

Plk-1-PBD Binding Probe



PubChem-SID 47212999,
PubChem-CID 5401876
Plk-1-PBD Binding IC_{50} 13.28 μM

Chemical name: N-(2-methoxyphenyl)-N-[(3-oxo-1-benzothiophen-2-ylidene) amino] acetamide

Molecular Formula:
$C_{17}H_{14}N_2O_3S$

Molecular Weight:
326.36966

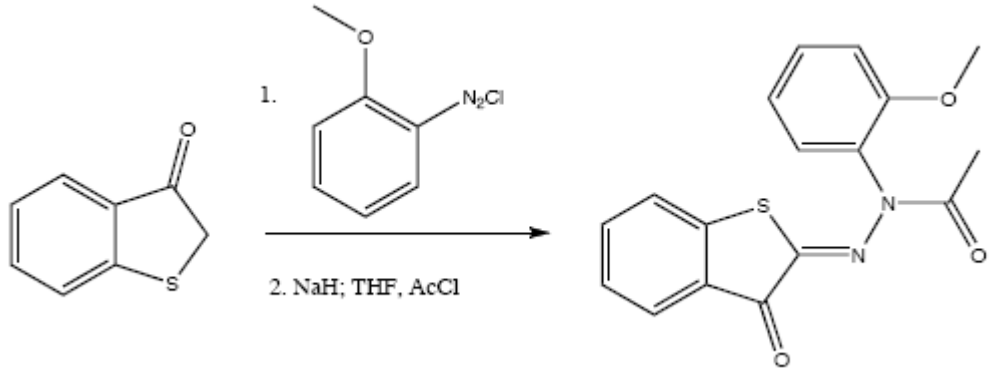
Describe mode of action for biological activity of probe:
No Mechanism of action Enzyme Kinetics data presently available

Has the compound been provided to the MLSMR: No, a sample of this compound was purchased on behalf of Dr Michael Yaffe (MIT) by Dr. David Weaver of DNAR from commercial sources (Source: Ambinter STOCK3S-55269; ZINC ZINC00227365) and provided to the PMLSC for testing.

Center comments on chemistry strategy leading to probe identification:
The probe compound PubChem-SID 47212999 is structurally related to two hits identified in the primary screen, PubChem-SID's 14742370 and 14742458 that are constituents of the NIH MLSMR. A number of related analogs were purchased from commercial vendors, and provided preliminary SAR.

Detailed synthetic pathway for making probe:

Figure 3. Synthetic Pathway for PubChem-SID 47212999



Center Summary of Probe Properties:

From PubChem:

XLogP: 3.4; Hydrogen Bond Donor Count: 0; Hydrogen Bond Acceptor Count: 4; Rotatable Bond Count: 3; Exact Mass: 326.072513; MonoIsotopic Mass: 326.072513; Topological Polar Surface Area: 59; Heavy Atom Count: 23; Charge: 0; Complexity: 508; Isotope Atom Count: 0; Defined Atom StereoCenter Count: 0; Undefined Atom StereoCenter Count: 0; Defined Bond StereoCenter Count: 1;Undefined Bond StereoCenter Count: 0; Covalently-Bonded Unit Count: 1

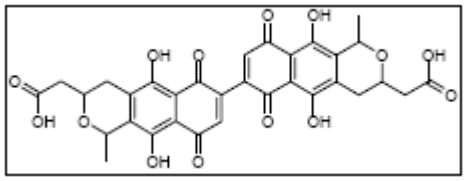
Leadscope Properties:

PubChem-SID	Parent Molecular Weight	ALogP	Hydrogen Bond Acceptors	Hydrogen Bond Donors	Lipinski Score	Molecular Weight	Parent Atom Count	Polar Surface Area	Rotatable Bonds	PLK-1-PBD IC50 uM
47212999	326.3686	3.35	4	0	0	326.3686	23	58.97	4	13.276

Appendices:

I. Comparative data on probe, similar compound structures and prior probes

Figure 4. Existing Plk-1-PBD Binding Inhibitor



NCI 293161

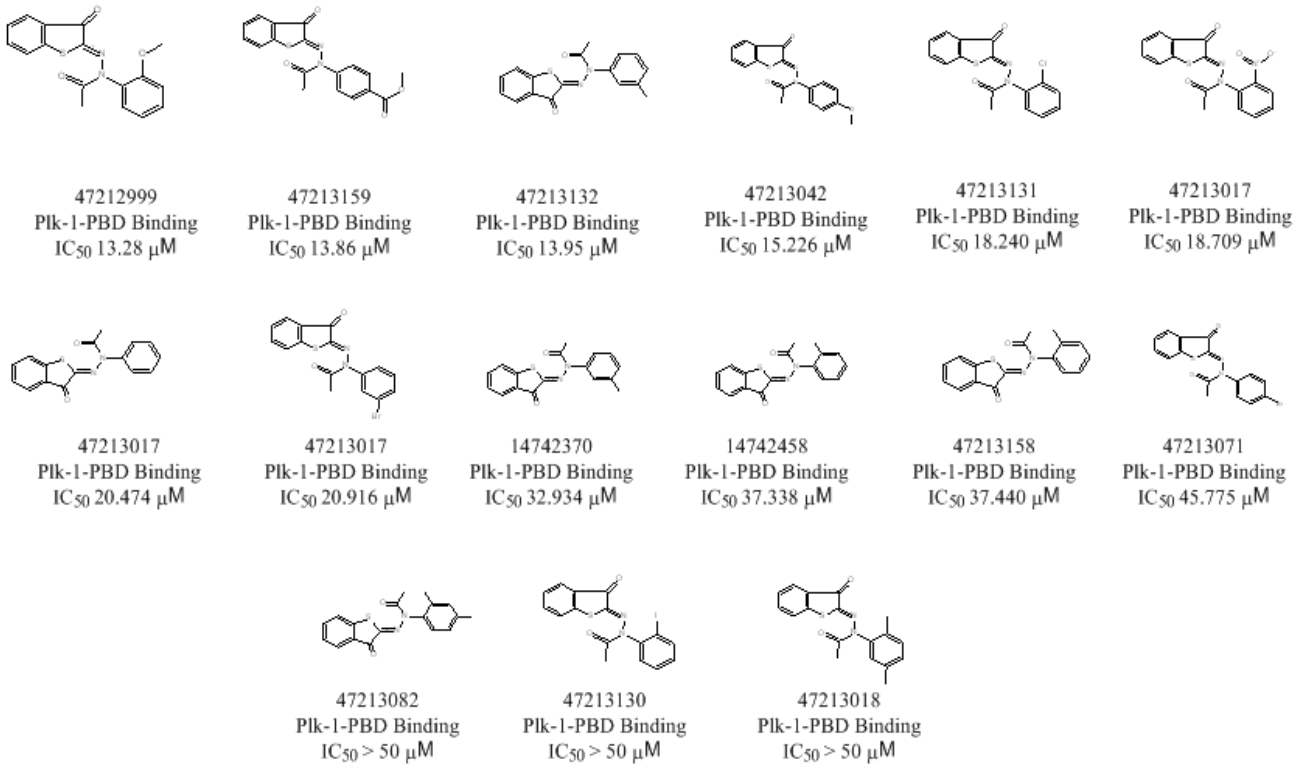
NCI 293161was identified in a small scale screen of 3362 compounds using the Plk-1-PBD fluorescence polarization binding assay. NCI 293161 was confirmed as a concentration dependent inhibitor in the Plk-1-PBD fluorescence polarization binding assay. NCI 293161 was active in a biochemical assay for inhibition of PBD binding to phospho-protein ligands, and in a biochemical assay using protein ligand binding (visualized by western blotting) as the readout. NCI 293161 affected the growth and morphology of U2OS cells relative to those of the negative control and DMSO treated cells. Treatment of U2OS cells with NCI 293161 resulted in a significant accumulation of rounded, refractile and non-adherent cells. NCI 293161 appeared to have a similar effect on proliferating MEFs as on U2OS cells. Interestingly, regions of the same well that contained a higher cell density and were therefore locally confluent, appeared unaffected by NCI 293161 treatment. Based on the Plk-1-PBD binding assay the IC50 for NCI 293161 was ~4 μM. However additional characterization in the Yaffe laboratory found that NCI 293161 arrested cells in S-phase, which is not consistent with Plk1 inhibition, but would be more consistent with some kind of DNA damaging effect.

II. Structure Activity Data on the Inhibition of Plk-1-PBD Binding by Related N-(2-methoxyphenyl)-N-[(3-oxo-1-benzothiophen-2-ylidene) amino] acetamide Compounds

Compounds were tested 10-point 2-fold dilution series concentration response curves run in singlicate starting at a maximum concentration of 50 μM (Figure. 5.).

Figure 5. Preliminary SAR data

Inhibition of Plk-1-PBD Binding by Related
N-(2-methoxyphenyl)-N-[(3-oxo-1-benzothiophen-2-ylidene) amino] acetamide Compounds



III. Cross Target Query of PubChem Database

A cross target query of the PubChem database with the two structurally related hits identified in the primary Plk-1-PBD screen and confirmed in concentration response assays, PubChem-SID's 14742370 and 14742458, revealed that these inhibitors were fairly selective. 14742370 and 14742458 have been tested in between 97 and 104 bioassays (Table 1), had only been flagged as active in 4 or 5 of those screens, and have only been confirmed against Plk-1-PBD.

Table 1. Cross target Query of PubChem for PubChem-SID's 14742370 and 14742458, PLK-1-PBD Binding Inhibitor Hits Related to the N-(2-methoxyphenyl)-N-[(3-oxo-1-benzothiophen-2-ylidene) amino] acetamide Compounds.

PubChem-SID	PLK-1-PBD IC50 μM	# Assays	# Active Flags	# Confirmed Actives	Confirmed Targets
14742370	32.93	104	4	1	PLK-1-PBD
14742458	37.34	97	5	1	PLK-1-PBD

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