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Development and Implementation of a Miniaturized High-Throughput Time-Resolved Fluorescence Energy Transfer Assay to Identify Small Molecule Inhibitors of Polo-Like Kinase 1

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Abstract

Polo-like kinase (Plk) 1 is a key enzyme involved in regulating the mammalian cell cycle that is also a validated anticancer drug target. Nonetheless, there are relatively few readily available potent and selective small molecule inhibitors of Plk1. To increase the availability of pharmacologically valuable Plk1 inhibitors, we describe herein the development, variability assessment, validation, and implementation of a 384-well automated, miniaturized high-throughput time-resolved fluorescence energy transfer screening assay designed to identify Plk1 kinase inhibitors. Using a small molecule library of pharmaceutically active compounds to gauge high-throughput assay robustness and reproducibility, we found nine general kinase inhibitors, including H-89, which was selected as the minimum control. We then interrogated a 97,101 compound library from the National Institutes of Health repository for small molecule inhibitors of Plk1 kinase activity. The initial primary hit rate in a single 10 μM concentration format was 0.21%. Hit compounds were subjected to concentration–response confirmation and interference assays. Identified in the screen were seven compounds with 50% inhibitory concentration (IC₅₀) values below 1 μM , 20 compounds with IC₅₀ values between 1 μM and 5 μM , and eight compounds with IC₅₀ values between 5 and 10 μM , which could be assigned to seven distinct chemotype classes. Hit compounds were also examined for their ability to inhibit other kinases such as protein kinase D, focal adhesion kinase, rho-associated coiled coil protein kinase 2, c-jun NH₂-terminal kinase 3, and protein kinase A via experimentation or data-mining. These compounds should be useful as probes for the biological activity of Plk1 and as leads for the development of new selective inhibitors of Plk1.

Introduction

Polo-like-kinase (Plk) 1 is a serine-threonine protein kinase that functions as a key regulator of mitosis/meiosis and cytokinesis.¹ Plk1 is expressed predominantly during the late G₂ and early M phase of the cell cycle, and, as cells pass through the various mitotic stages, Plk1 displays a dynamic pattern of subcellular localization to multiple mitotic structures.^{2,3}

Specifically, Plk1 is primarily localized at the centrosome during interphase and early prophase and then relocates to the kinetochores and midbody during metaphase and late mitosis, respectively.¹ The fluid nature of Plk1 localization to various mitotic structures directly affects Plk1 proximity to substrates or interacting proteins, such as Cdc25C, rho-associated coiled coil protein kinase 2 (ROCK2), Wee1, PICH, and BubR1,^{4–8} illustrating the complex nature of Plk1 in intracellular signaling.

Plk1 gene expression is frequently up-regulated in human cancers and carcinoma-derived cell lines, including pancreatic, ovarian, breast, non-small cell lung carcinoma, prostate, head and neck, esophageal and gastric, melanoma, colorectal, endometrial, glioma, and thyroid cancers.^{9–12} As a result Plk1 is considered a potential prognostic indicator for several types of cancers.^{13–17} Interest in Plk1 as a therapeutic target developed when Plk1 overexpression was found to induce transformation of National Institutes of Health (NIH)/3T3 fibroblasts and tumor progression in nude mice and Plk1-specific antisense oligonucleotides, short interfering RNA, or short hairpin RNA were found to decrease tumor cell survival and inhibit tumor growth in animal models.^{18–23} Taken together, these data help validate Plk1 as a rational anticancer drug target. Moreover, there is accumulating evidence that Plk1 may be an appropriate molecular target for other human diseases. For example, Plk1 is (1) expressed at elevated levels in synoviocytes from patients with rheumatoid arthritis and Plk1 short interfering RNA suppresses their proliferation, (2) detectable in neurons from patients with Alzheimer's disease but not from unaffected individuals, (3) up-regulated in CD4⁺ T cells from rhesus macaques infected with high viral load simian immunodeficiency virus and in primary human keratinocytes infected with human papillomavirus type 16 embryonic days 6 and 7, and (4) physically associates with and phosphorylates the human cytomegalovirus pp65 lower matrix protein upon infection of human fibroblasts and HeLa cells.^{24–28} These latter findings indicate Plk1 may be an important regulator in human disease mechanisms other than cancer and that Plk1 functionality can be exploited by pathogenic viruses.

Despite the attractiveness of Plk1 as a therapeutic target, there are only nine reported Plk1 inhibitors, almost all with some pharmacological or chemical liability. Wortmannin inhibits Plk1 with a 50% inhibitory concentration (IC₅₀) value of 24 nM, but it also inhibits phosphatidylinositol-3 kinase with an IC₅₀ of 5 nM and has a biological half-life of approximately 10 min, which limits its pharmacological applications.^{29,30} Staurosporine, scytonenim, purvalanol A, LY294002, morin, and quercetin inhibit Plk1 but have well documented cross-target effects and have IC₅₀ values ranging from approximately 2 to 65 μ M.^{29,31–34} In comparison, the Plk1 kinase inhibitors ON01910 and BI 2536 have IC₅₀ values for Plk1 of 10 and 0.80 nM, respectively, and both compounds are currently in Phase I clinical trials.^{35–38} However, ON01910 also inhibits CDK1 (with an IC₅₀ range of 18–269 nM) as well as additional kinases including Flt-1, platelet-derived growth factor receptor, and Abl at low nanomolar concentrations as well as the kinase activity of Src, Fyn, and Plk2 kinases at higher concentrations.³⁵ In contrast, BI 2536 has been reported to be a selective and potent Plk1 inhibitor.^{38,39} Unfortunately, neither ON01910 nor BI 2536 is readily available to the general scientific community.

The purpose of the study described herein was to develop, validate, and implement an HTS assay that could be used to interrogate compound libraries for small molecule inhibitors of Plk1 kinase activity. The assay format selected for HTS assay development was the immobilized metal assay for phosphochemicals (IMAP™) time-resolved fluorescence resonance energy transfer (TR-FRET) technology by Molecular Devices (Sunnyvale, CA). In general, the IMAP technology is a generic, homogeneous, mix-and-read assay format that can be implemented to quantify kinase activity, and it is based on a specific association of trivalent, metal-containing nanoparticles with phosphate moieties.^{40,41} These phosphate groups are generated in kinase reactions consisting of enzyme, ATP, and fluorescently labeled substrate, and addition of the IMAP nanoparticle-based binding buffer stops the kinase reaction and binds phosphorylated, fluorescently labeled substrates. This phosphosubstrate-nanoparticle binding event can be quantified by either fluorescence polarization (FP) or TR-FRET. With IMAP FP, the complexing of the phosphosubstrate with the nanoparticle decreases the motion of the phosphosubstrate, and when this large complex is excited by polarized light, the emitted light is polarized, resulting in an increase in measured millipolarization units.⁴¹ By comparison, with IMAP TR-FRET, the nanoparticle-containing binding buffer is supplemented with a terbium (Tb) donor molecule, which is also capable of binding to the nanoparticle. A complexing of the phosphosubstrate, nanoparticle, and Tb donor molecule brings the fluorescently labeled phosphosubstrate in close proximity to the Tb donor.⁴² Excitation of the Tb donor results in a transfer of energy to the phosphosubstrate, which in turn fluoresces at its specified wavelength. This allows for the measurement of the TR-FRET between the Tb donor and the phosphorylated, fluorescent substrate.

The identification of readily available small molecule chemical inhibitors of Plk1 should provide the scientific community at large with (1) a larger inhibitor “toolbox” to help delineate the functional role of Plk1 at the molecular level and (2) the basis for future analog design and synthesis for drug discovery targeting many disease model systems. Reported here are the results from a 384-well, miniaturized TR-FRET HTS assay that was used to screen a 97,101 compound NIH Small Molecule Repository, supplemented with secondary screening, kinase specificity, and lead identification data.

Materials and Methods

Chemicals, assay reagents, and supplies

White, opaque, small-volume microtiter plates were purchased from Greiner (Monroe, NC) and used for all experiments. The IMAP progressive binding reagent, binding buffer (pH ~5.5), kinase reaction buffer (10 mM Tris-HCl [pH 7.2], 10 mM MgCl₂, 0.05% NaN₃, 1 mM dithiothreitol [DTT], and 0.01% Tween-20) and substrate peptide (5-carboxyfluorescein-KKRNRRRLSVA-OH) were obtained from Molecular Devices. Kinase-active glutathione *S*-transferase (GST)-tagged recombinant Plk1 was obtained from Cell Signaling Technologies (Danvers, MA). ATP was purchased from GE Healthcare (Piscataway, NJ). H-89 and Gö6976 were obtained from Millipore (Billerica, MA) and VWR (Bridgeport, NJ), respectively. Dimethyl sulfoxide (DMSO) and the library of pharmacologically active compounds (LOPAC) were purchased from Sigma-Aldrich (St.

Louis, MO). The 97,101 compound library screened for Plk1 small molecule inhibitors was made available by the Pittsburgh Molecular Libraries Screening Center (PMLSC) (Pittsburgh, PA) as part of the NIH Molecular Libraries Screening Centers Network (MLSCN) Roadmap Initiative. Cherry-picked compounds from the PMLSC library were supplied by BiofocusDPI (a Galapagos Company, San Francisco, CA).

Western blotting and silver staining

Purified Plk1 protein samples were separated by electrophoresis using 10% Bis-Tris NuPAGE® polyacrylamide gels (Invitrogen, Carlsbad, CA). For western blotting procedures, gels were transferred to 0.45- μ m (pore size) nitrocellulose membranes (Whatman, Dassel, Germany) and probed with Plk1 (1 μ g/ml) or GST-tag anti-bodies (1 μ g/ml). The protein/antibody complex was visualized using anti-rabbit immunoglobulin G horseradish peroxidase-linked antibody (1 μ g/ml) from Jackson Laboratories (Bar Harbor, ME) and enhanced chemiluminescence western blotting (GE Healthcare). For silver staining procedures, gels were processed using the SilverExpress® staining kit (Invitrogen) according to the manufacturer's recommendations.

IMAP TR-FRET assay protocol for assay development and HTS

The Plk1 HTS screening assay was formatted using miniaturized 6- μ l kinase reaction volumes in a 384-well microtiter plate format. For HTS procedures, each assay component was added to individual wells using a Velocity (Menlo Park, CA) V-prep liquid handling system equipped with a 384-well dispensing head. Plk1 kinase reactions were assembled by stepwise addition of 2 μ l of substrate/ATP, compound (or control reagent), and Plk1 enzyme 3 $\times$ working solutions followed by centrifugation at 500 g for 1 min. Negative (MAX) controls contained 1% DMSO, and positive (MIN) controls contained 100 μ M H-89 in 1% DMSO (final concentrations). Plk1, substrate peptide/ATP, and compounds (or control reagent) were prepared in kinase reaction buffer (10 mM Tris-HCl [pH 7.2], 10 mM MgCl₂, 0.05% NaN₃, 1 mM DTT, and 0.01% Tween-20). Tb-only and buffer-only controls were also prepared. The final concentrations of substrate/ATP, Plk1, and compounds/controls were 750 nM/25 μ M, 100 mU/ml, and 10 μ M, respectively, unless otherwise stated in individual assay development procedures. The kinase reaction was allowed to proceed for 3 h at room temperature (unless stated otherwise), and the reaction was stopped with addition of 18 μ l of IMAP binding reagent (1:400) in 1 $\times$ IMAP progressive binding buffer (70% buffer A/30% buffer B) containing a 1:600 dilution of Tb. The binding buffers A and B used in this assay were obtained from Molecular Devices, and the composition of the binding buffers is proprietary.⁴³ Assay plates were centrifuged at 500 g for 1 min and then allowed to incubate at room temperature for a minimum of 5 h, unless otherwise stated. TR-FRET data were captured on a Molecular Devices SpectraMax M5 (excitation Tb A₃₃₀; emission Tb A₄₉₀; emission 5-carboxyfluorescein acceptor A₅₂₀). TR-FRET corrected units were calculated as follows:

$$\frac{\text{Background-subtracted } A_{520} - (\text{P factor} \times \text{background-subtracted } A_{490})}{\text{Background } A_{490}}$$

$$\text{Background-subtracted } A_{490} = \text{Tb-only } A_{490} - \text{buffer-only } A_{490}$$

where

$$\text{Background-subtracted } A_{520} = \text{Tb-only } A_{520} - \text{buffer-only } A_{520}$$

and

$$\text{P factor} = \frac{\text{background-subtracted } A_{520}}{\text{background-subtracted } A_{490}}$$

Library compound dilution scheme for primary screening and 10-point IC₅₀ determinations

In primary screening activities, 2 μl of 1 mM test compounds in 100% DMSO was diluted in 64.7 μl of 1 $\times$ kinase reaction buffer creating a 30 μM working concentration of library compounds. Upon assembly of all kinase reaction components (substrate/ATP, Plk1, and compound), the final test compound concentration was 10 μM . In 10-point IC₅₀ determination experiments, 2 μl of 10 mM test compounds in 100% DMSO were diluted in 133.3 μl of 1 $\times$ kinase reaction buffer creating a 150 μM working concentration of library compounds. A twofold serial dilution was then performed creating a threefold concentration range (0.3–150 μM). Upon assembly of all kinase reaction components the final 10-point concentration range was 0.1–50 μM .

Determination of compound interference with the TR-FRET assay format

Hit compounds were screened for interference with the IMAP TR-FRET assay format. Briefly, Plk1 kinase reactions were allowed to proceed in the absence of compounds as described above. Compounds (final concentration 10 μM) were then added to the kinase reactions. IMAP binding reagent (1:400) in 1 $\times$ binding buffer (70% buffer A/30% buffer B) containing a 1:600 dilution of Tb was then added to each microtiter plate well. TR-FRET data were captured on a SpectraMax M5 (excitation Tb A_{330} ; emission Tb A_{490} ; emission 5-carboxyfluorescein acceptor A_{520}) as described above.

IMAP-based protein kinase D (PKD) FP assay

Hit compounds were assayed for their ability to inhibit PKD using an IMAP-based PKD FP assay. The PKD FP assay was performed in a miniaturized reaction volume (6 μl) and followed the same basic protocol as described for the Plk1 TR-FRET assay. Briefly, PKD kinase reactions were assembled by stepwise addition of 2- μl volumes of 3 $\times$ working concentrations of substrate/ATP (300 nM/60 μM), compound, and PKD enzyme (0.18 units/ml) followed by centrifugation at 500 g for 1 min. Negative (MAX) controls contained 1% DMSO, and positive (MIN) controls contained 1 μM Gö6976 in 1% DMSO (final concentrations).⁴⁴ Reagents were prepared in kinase reaction buffer (10 mM Tris-HCl [pH 7.2], 10 mM MgCl₂, 0.05% NaN₃, 1 mM DTT, and 0.01% bovine serum albumin). The kinase reaction was allowed to proceed for 90 min at room temperature, and the reaction was

stopped with addition of 18 μ l of IMAP binding reagent (1:400) in 1 $\times$ binding buffer A. Assay plates were centrifuged at 500 *g* for 1 min and then allowed to incubate at room temperature for 2 h. FP data were captured on a SpectraMax M5 (excitation A_{485} ; emission A_{520}).

HTS data analysis, visualization, and statistical analysis

HTS assay development and validation data were analyzed using University of Pittsburgh Drug Discovery Institute (UPDDI) software. HTS data analysis for primary screening and IC₅₀ determinations was performed using ActivityBase™ (IDBS, Guildford, UK), CytoMiner (UPDDI), and Spotfire® (Somerville, MA) software. The Leadscope (Columbus, OH) Predictive Data Miner software was used to analyze confirmed hit compounds. Additional statistical and data analyses were performed using GraphPad (San Diego, CA) Prism software version 4.0. The Pubchem database (<http://pubchem.ncbi.nlm.nih.gov>) was used to identify compounds that displayed inhibitory effects on additional kinases.

Results

Development of a high-throughput assay to screen for Plk1 inhibitors

Our initial experiments focused on characterizing the Plk1 protein that was used during assay development and HTS procedures. In preliminary experiments we determined the half-maximal TR-FRET signal with this assay format was 100 mU/ml (0.6 mU/6 μ l of reaction volume) (Fig. 1A). The purity of the GST-tagged Plk1 preparation was demonstrated by the predominant protein band at the predicted 100 kDa molecular mass in the silver-stained gel as well as in the anti-Plk1 and anti-GST western blots (Fig. 1B). Subsequent studies focused on determining the linearity of Plk1 enzymatic response over time. Increasing Plk1 concentrations were incubated with ATP (25 μ M) and substrate peptide (750 nM) at room temperature for the indicated times (Fig. 1C). We stopped the kinase reactions by adding the binding reagent/Tb buffer, and we captured the data after a 1-h incubation at room temperature. A time course was also performed without Plk1 as a control. The enzymatic responsiveness of the Plk1 was linear over a 180-min kinase reaction time at each of the Plk1 concentrations (37, 100, and 170 mU/ml) tested ($R^2 > 0.93$). We selected 100 mU/ml Plk1 ($R^2 > 0.99$) for further assay development procedures because it produced a reasonable starting signal to background (S:B) ratio that could be enhanced with further assay optimization.

We next determined the K_m values for ATP and substrate peptide. The K_m for ATP ($K_{m,ATP}$) was calculated by varying the ATP concentration (0–300 μ M) in the kinase reaction while maintaining constant Plk1 (100 mU/ml) and substrate peptide (100 nM) concentrations. Kinase reactions and data acquisition were performed as described above. The $K_{m,ATP}$ ($7.8 \pm 3.1 \mu$ M) was calculated from three independent experiments and was similar to previously published values (Fig. 2A).^{35,45} We elected to use an excess of ATP (25 μ M) in further assay development procedures. Similarly, the K_m for substrate peptide ($K_{m,substrate}$) was calculated by increasing the peptide concentration (0–4 μ M) within the kinase reaction while keeping the Plk1 (100 mU/ml) and ATP (25 μ M) concentrations constant. Kinase reactions and data acquisition were performed as described above. Based on the $K_{m,substrate}$ (742 ± 70 nM) ($n =$

3) (Fig. 2B), we selected a substrate peptide concentration of 750 nM (approximate $K_{m, \text{substrate}}$) for further assay development procedures.

To establish a robust IMAP TR-FRET automated HTS assay, we examined additional parameters such as enzyme stability and pH optimum for the enzyme and characterized the HTS assay control reagents. Figure 3 illustrates Plk1 stability under different handling conditions. Plk1 enzyme aliquots were stored on ice or at room temperature, in concentrated and diluted solutions, for the indicated times (Fig. 3A and B). Plk1 activity was stable for up to 4 h on ice when the enzyme was concentrated (*i.e.*, not diluted to its working concentration) (Fig. 3A). In contrast, when Plk1 was stored diluted on ice (Fig. 3A) or at room temperature (concentrated and diluted) its enzymatic activity decreased significantly after 1 h (Fig. 3B) (analysis of variance). Plk1 enzymatic activity was stable after eight freeze-thaw cycles (Fig. 3C). The assay tolerated up to 1.25% (vol/vol) DMSO without the loss of signal (data not shown).

Our selected HTS assay MIN control was H-89. Figure 4A shows that H-89 inhibited Plk1 with an IC_{50} of $4.9 \pm 1.9 \mu M$ ($n = 3$ independent experiments). Additional studies demonstrated that H-89 did not interfere with the IMAP TR-FRET assay format (data not shown) and provided a reasonable (fourfold) signal window. Based on these data, we used 100 μM H-89 as our HTS assay MIN control. Studies designed to characterize the pH optimum of the Plk1 in the selected buffer composition determined that no significant difference in assay readout occurred over a pH range of 6.0–8.5 (Fig. 4B) (analysis of variance). Thus, subsequent HTS assays were performed at pH 7.2 to maintain physiologically relevant assay conditions. Lastly, the maximal TR-FRET readout was observed after 5 h of incubation with binding/Tb buffer, and this maximal signal was maintained for up to 16 h (data not shown). Therefore, assay plates were allowed to incubate with binding reagent for 5 h prior to data collection.

Three-day variability assessment procedures confirmed suitability of Plk1 TR-FRET assay for HTS

To demonstrate the suitability of the TR-FRET assay for HTS, we performed a 3-day variability assessment that consisted of running two plates as MAX controls and two plates as MIN controls in three independent trials (for a total of 12 plates). Figure 5 shows the scatter plots from the three testing days. Results from the 3-day variability assessment demonstrated that the assay had an average signal window of 3.8 ± 0.2 and Z -factor of 0.6 ± 0.2 . Coefficients of variation were below 10%; however, a process error with the fourth plate resulted in higher standard deviations (SDs) and a lower Z -factor in one data set (*i.e.*, Z -factor = 0.41). Therefore, we instituted precautions for future HTS assay setups including increasing the void volumes and limiting the number of assay plates to be set up at one time to four or five (*i.e.*, batch processing).

LOPAC library screening confirmed HTS assay reproducibility

The ability of the Plk1 HTS TR-FRET assay to detect the effects of test compounds was demonstrated by screening the LOPAC library. Each test compound was assayed at a single concentration of 10 μM (with a final concentration of 1% DMSO) to reproduce actual

screening conditions (1,280 compounds, 320 per plate). The LOPAC library was screened in duplicate, and a hit compound was defined as any compound that resulted in $\geq 50\%$ inhibition of Plk1 TR-FRET MAX signal. A total of 30 hit compounds were identified, including nine known kinase inhibitors (data not shown). Significantly, our selected HTS assay MIN control, H-89, was one of the nine known kinase inhibitors ($81.4 \pm 2.9\%$ inhibition) identified as a hit compound in the screen. Overall, the screen of the LOPAC library displayed a 2.3% hit rate with the reproducibility between duplicate assays being represented in Fig. 6 (calculated R^2 value of 0.94). There was considerable agreement for the Z -factors and S:B ratios between the duplicate LOPAC screenings: 0.64 ± 0.01 versus 0.65 ± 0.06 and 4.20 ± 0.11 versus 4.24 ± 0.13 , respectively.

Plk1 HTS primary screen of the PMLSC compound library and secondary hit characterization

After confirming the reproducibility of the Plk1 HTS TR-FRET assay, we screened the PMLSC 97,101 compound library for small molecule inhibitors of Plk1 using the optimized assay conditions. Each test compound was assayed at a single concentration of $10 \mu\text{M}$ (final concentration, 1% DMSO), and any compound that resulted in $\geq 70\%$ inhibition of Plk1 TR-FRET MAX signal was considered a hit compound. The initial primary screen identified 209 Plk1-inhibiting compounds for a 0.21% hit rate. Under the optimized HTS conditions, the average Z -factor and S:B ratio for the screen of the 97,101 compound library (~ 304 384-well microtiter plates) were 0.66 ± 0.09 and 4.66 ± 0.43 , respectively (Fig. 7). Four assay plates had Z -factors significantly below 0.5; however, after data analysis using Spotfire, it was determined that the low Z -factors were due to one outlier in the MAX control wells of each respective assay microtiter plate. Therefore, the assay plates were passed.

We interrogated the 209 compounds in a series of secondary screening procedures that included a repeat of the primary screen, an evaluation for TR-FRET assay format interference, an IC_{50} determination, and a kinase specificity assay. The primary screen was repeated in duplicate, and compounds were considered confirmed if they exhibited $\geq 50\%$ inhibition in all three primary screening assays. Using these criteria, 42 compounds were confirmed as hit compounds, two of which, Pubchem substance identity number (SID) 17409262 and 14745671, interfered ($\geq 50\%$ inhibition of MAX signal) with the IMAP TR-FRET assay format resulting in an overall 19.1% hit confirmation rate.

The 40 hit compounds were assayed in duplicate with compound concentration ranges of 0.1 – $50 \mu\text{M}$ (Fig. 8). Seven compounds exhibited average IC_{50} concentrations below $1 \mu\text{M}$, 20 compounds had IC_{50} values 1 – $5 \mu\text{M}$, eight compounds had IC_{50} values 5 – $10 \mu\text{M}$, and five compounds had IC_{50} values $> 10 \mu\text{M}$. Leadscape analysis identified seven structural clusters, although the majority (15 of 40) of hit compounds were not computationally assigned to a structural cluster and were classified as singletons. Hit compounds were also initially examined for their ability to inhibit the activity of other kinases by implementing an IMAP-based HTS PKD FP assay and data-mining the Pubchem database (<http://pubchem.ncbi.nlm.nih.gov>). Thirty-nine compounds displayed no inhibition against PKD with an $\text{IC}_{50} > 50 \mu\text{M}$, while one compound, Pubchem SID 17407044, inhibited PKD kinase activity with an IC_{50} value of $8.0 \mu\text{M}$. By comparison, Pubchem SID 17407044

inhibited Plk1 kinase activity with an IC_{50} value of $0.2 \pm 0.02 \mu M$. When the Pubchem database was mined, 39 compounds were either inactive or lacked reproducible inhibition in other kinase assay screens, including those for focal adhesion kinase (FAK), c-jun NH₂-terminal kinase (JNK) 3, ROCK2, and protein kinase A (PKA); however, Pubchem SID 852914 (Plk1 $IC_{50} = 4.2 \pm 1.1 \mu M$) was confirmed as a ROCK2 and PKA inhibitor in concentration–response studies with reported IC_{50} values of 0.8 and 11 μM , respectively (<http://pubchem.ncbi.nlm.nih.gov>).

Discussion

We developed a 384-well miniaturized HTS assay to interrogate compound libraries for novel small molecule Plk1 kinase inhibitors. Reported here are the assay development and implementation results from the screening of a 97,101 compound library made available as part of the NIH Roadmap Initiative for Molecular Libraries and Imaging. Specific assay parameters were empirically determined to ensure an appropriately robust HTS assay with respect to *Z*-factors, S:B ratios, and identification of assay control compounds. After implementation of the optimized and validated HTS assay procedures a total of 209 actives were identified from primary screening activities (0.21% hit rate). Through a series of secondary screening assays, 40 hit compounds were identified as small molecule Plk1 inhibitors. Concentration–response studies of the 40 hit compounds revealed IC_{50} values with a majority (67.5%) having IC_{50} values $<5 \mu M$ against Plk1 kinase activity. Initial specificity studies, using an HTS PKD FP assay, indicated that the inhibition of Plk1 by the hit compounds was specific, with only one compound (2.5% of total confirmed hit compounds) inhibiting PKD in a kinase counterscreen.

We selected the IMAP-based technology as a basis for our TR-FRET assay because the technology platform is highly amenable to HTS assay development and implementation and can be efficiently and effectively miniaturized to 384- and 1,536-well formats.^{40,41,46–48} Moreover, the TR-FRET assay format is superior to its companion IMAP FP format with respect to following Michaelis-Menten enzyme kinetics and classic assay development procedures.^{49,50} We performed the Plk1 TR-FRET assay at higher than $K_{m,ATP}$ concentrations (25 μM vs. $\sim 8.0 \mu M$) primarily to achieve a reasonable assay signal window in the fluorescence-based assay format. However, we also wanted to bias assay outcomes against the identification of ATP analogues. Because the TR-FRET readout emanates from the substrate peptide and does not strictly rely on ATP consumption, it is not explicitly affected by ATP concentrations.⁴⁶ This is in direct contrast to an assay format that quantifies kinase activity through the consumption of ATP (such as a chemiluminescent kinase assay like Kinase-Glo™ [Promega, Madison, WI]). Therefore TR-FRET allows for flexibility in ATP concentrations. Significantly, as indicated in Fig. 1C, higher ATP concentrations (25 μM) did not jeopardize the linearity of the Plk1 enzymatic reaction.

Most identified Plk1 kinase inhibitors, including staurosporine, wortmannin, LY294002, morin, quercetin, and BI 2536, are ATP-competitive inhibitors. However, scytonemin displays both ATP- and non-ATP-competitive characteristics, while ON01910 is a non-ATP-competitive Plk1 inhibitor, most likely binding near the peptide binding site.^{17,33,35} We screened staurosporine, scytonemin, wortmannin, and H-89 within our HTS assay system to

identify an HTS assay MIN control. Because we used an excess of ATP in the reaction, it was not surprising that calculated IC₅₀ values were very high since all of the compounds tested are ATP competitors or displayed ATP-competitive characteristics. For example, inhibition of Plk1 kinase activity with staurosporine in our assay would not consistently reproduce IC₅₀ values in the low micromolar range as previously reported (data not shown).³² In contrast, H-89 reproducibly inhibited Plk1 activity, as measured by TR-FRET signal, with an IC₅₀ value of ~5 μ M.

To date, BI 2536 appears to be the only Plk1 inhibitor that exhibits some degree of specificity as well as significant potency. However, there is evidence that BI 2536 inhibits the activity of Plk2 and Plk3.^{17,39} The high degree of structural homology between ATP binding pockets makes the identification of specific kinase inhibitors challenging, especially among kinase isoforms. This suggests it may be advantageous to bias the identification of Plk1 kinase inhibitors against ATP-competitive inhibitors, and our preliminary data reported here support this strategy. Specifically, when compared structurally with ATP, none of the 40 hit compounds identified had Tanimoto similarity scores >0.5, and only two compounds (Pubchem SID 863266 and 17403478) had Tanimoto similarity scores of 0.3. Initial data also suggest that the 40 hits are selective Plk1 inhibitors. The majority of Plk1 small molecule inhibitors identified with this assay did not inhibit PKD, and data-mining of the Pubchem database demonstrated the majority of the identified Plk1 small molecule inhibitors did not inhibit FAK, JNK3, ROCK2, and PKA in other concentration–response HTS kinase assays. Moreover, the inability of the majority of PLK1 small molecule inhibitors to inhibit the activity of PKD was confirmed with the implementation of the IMAP FP PKD HTS assay against the NIH Small Molecule Repository.⁵¹ The only compound that registered as being inhibitory against PKD in concentration–response studies was Pubchem SID 17407044 (data not shown), confirming our original data. However, significant lead characterization studies are necessary in order to understand the mechanism of action and the specificity of the identified hit compounds.

In summary, an automated HTS Plk1 TR-FRET assay has been developed, validated, and used for the rapid identification of small molecule Plk1 kinase inhibitors. We used the HTS Plk1 TR-FRET assay to screen the PMLSC library, which consisted of 97,101 individual compounds. Forty Plk1 small molecule inhibitors were identified and are now progressing through a series of lead characterization procedures including additional kinase specificity assays, data-mining via Pubchem, radio-metric Plk1 kinase assays, ATP competition assays, and a cell-based bipolar spindle assay.

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ABBREVIATIONS:

DMSO	dimethyl sulfoxide
DTT	dithiothreitol

FAK	focal adhesion kinase
FP	fluorescence polarization
GST	glutathione <i>S</i> -transferase
IC₅₀	50% inhibitory concentration
IMAPTM	immobilized metal assay for phosphochemicals (Molecular Devices, Sunnyvale, CA)
JNK3	c-jun NH ₂ -terminal kinase
LOPAC	library of pharmacologically active compounds
MLSCN	Molecular Libraries Screening Centers Network
NIH	National Institutes of Health
Plk	polo-like kinase
PMLSC	Pittsburgh Molecular Libraries Screening Center
PKA	protein kinase A
PKD	protein kinase D
ROCK2	rho-associated coiled coil protein kinase 2
S:B	signal to background
SD	standard deviation
SID	substance identity number
Tb	terbium
TR-FRET	time-resolved fluorescence resonance energy transfer
UPDDI	University of Pittsburgh Drug Discovery Institute

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FIG. 1.

Initial characterization of Plk1. **(A)** Confirmation of Plk1 activity in the TR-FRET assay format with a calculated half-maximal value of 100 mU/ml. **(B)** Silver staining and immunoblotting Plk1 using anti-Plk1 and anti-GST antibodies. **(C)** Linearity of Plk1 activity over 180 min: 170 mU/ml (▼), 100 mU/ml (■), 37 mU/ml (◇), and no enzyme control (●). Rfu, relative fluorescence units.

FIG. 2.

Plk1 kinetic constant determinations for ATP and substrate peptide. **(A)** $K_{m,ATP}$ was calculated as $7.8 \pm 3.1 \mu M$. **(B)** $K_{m,substrate}$ was calculated as $742 \pm 70 nM$. Both K_m calculations were obtained using GraphPad Prism version 4.0 ($n = 3$ independent experiments for each determination $\pm$ SD). Rfu, relative fluorescence units.

FIG. 3.

Stability of Plk1 activity under different handling conditions. **(A)** Plk1 was stored on ice in concentrated (black column) or diluted (gray column) solution and assayed for activity at specific intervals over a 4-h period. The white column indicates the no enzyme control. **(B)** Plk1 was stored at room temperature in concentrated (black column) or diluted (gray column) solution and assayed for activity at specific intervals over a 4-h period. The white column indicates the no enzyme control. **(C)** Concentrated Plk1 was subjected to eight freeze-thaw cycles and assayed for stability of enzymatic response as measured by TR-FRET (corrected)/Tb relative fluorescence units (Rfu) $\times$ 10,000 signal. The black column represents complete kinase reactions, and the white bar represents the no enzyme controls. The bars represent the SD from three independent determinations.

FIG. 4.

H-89 inhibitor IC_{50} and pH optimum determinations. **(A)** Plk1 kinase reactions were performed in triplicate using the HTS optimized conditions and assayed in the presence of varying concentrations of H-89. Each curve line represents an independent experiment, and data yielded an average IC_{50} value for H-89 of $4.9 \pm 1.9 \mu$ M. **(B)** Plk1 kinase reactions were performed over a range of buffer pH (6.0–8.5). Kinase response was stable across the pH range as measured by TR-FRET (corrected)/Tb relative fluorescence units (Rfu) $\times 10,000$ signal and analyzed by one-way analysis of variance (black bar). A no enzyme control (white column) and a 100 μ M H-89 MIN control (gray column) were assayed in parallel. The bars represent the SD from three independent determinations.

FIG. 5.

Three-day variability assessments of optimized HTS procedures. (A–C) Performance of the Plk1 TR-FRET assay in an automated 384-well HTS format was demonstrated by running two full MAX and MIN control plates on three independent assay days. From these data *Z*-factors, S:B ratios, and coefficients of variation were determined and reflected the Plk1 TR-FRET HTS assay's robustness using compound libraries. The solid and dashed lines represent ± 3 SD. Solid black and gray squares represent MAX plates 1 and 2, respectively. Solid white squares and diamonds represent MIN plates 1 and 2, respectively. Rfu, relative fluorescence units.

FIG. 6.

Comparison of two independent LOPAC screening runs. HTS assay reproducibility was evaluated by comparing two data sets collected from screening the LOPAC compound library, in duplicate. The results were plotted as a scatterplot. All data points are shown including MAX (open square), MIN ($\times$), and compounds (solid circle). The calculated R^2 value was 0.94.

FIG. 7.
 Z' -factors (■) and S:B (◇) ratio distribution for the NIH Small Molecule Repository library screen of 304 384-well microtiter plates.

A

B

Cluster 1

Cluster 2

FIG. 8.

Structures and IC_{50} determinations for the 40 Plk1 kinase inhibitors identified in the PMLSC library screen. IC_{50} values were calculated after Plk1 kinase reactions were exposed to a concentration range (0.1–50 μM) of compound in duplicate runs. The ActivityBase software package was used to perform the calculations, and active compounds were subjected to structure-based clustering using Leadscape Enterprise version 2.4.6–1 software. Singleton structures (**A**) and clusters of related structures (**B**) are presented with Pubchem SIDs, Plk1 IC_{50} values (in μM), and chemical structures.