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Wortmannin, a Widely Used Phosphoinositide 3-Kinase Inhibitor, also Potently Inhibits Mammalian Polo-like Kinase

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Summary

Polo-like kinases (PLKs) play critical roles throughout mitosis. Here, we report that wortmannin, which was previously thought to be a highly selective inhibitor of phosphoinositide (PI) 3-kinases, is a potent inhibitor of mammalian PLK1. Observation of the wortmannin-PLK1 interaction was enabled by a tetramethylrhodamine-wortmannin conjugate (AX7503) that permits rapid detection of PLK1 activity and expression in complex proteomes. Importantly, we show that wortmannin inhibits PLK1 activity in an in vitro kinase assay with an IC_{50} of 24 nM and when incubated with intact cells. Taken together, our results indicate that, at the concentrations of wortmannin commonly used to inhibit PI 3-kinases, PLK1 is also significantly inhibited.

Introduction

Polo-like kinases (PLKs) play many important roles during mitosis, as they are involved in the activation of cyclin B/Cdc2 through Cdc25C phosphatase [1–4], chromosome separation [5–8], centrosome maturation [8–10], bipolar spindle assembly [6, 10], regulation of the anaphase-promoting complex [11, 12], and execution of cytokinesis [13, 14]. Expression of PLK protein is restricted to proliferating cells, and PLK protein levels are regulated during the cell cycle. In mammalian cells, the PLK1 mRNA level is low in G1/S, starts to rise in G2, reaches maximal in mitosis, and then drops to the low basal level seen in G1 phase [15, 16]. In addition to this transcriptional control, the kinase activity of PLK is also regulated during the cell cycle. Mammalian PLK1 activity is stimulated by phosphorylation on threonine 210 [17].

Human PLK1 has been shown to be overexpressed in various human cancers, including head and neck squamous cell carcinomas, non-small cell lung cancer, oropharyngeal carcinomas, melanomas, ovarian and endometrial carcinomas, and prostate cancer [18–21]. Consequently, PLK1 has been proposed as a diagnostic marker for several different types of cancers [18–21]. Additionally, constitutive expression of PLK1 is able to transform mouse NIH-3T3 cells and induces tumor growth in nude mice [22]. Recent studies with small interfering RNA (siRNA) demonstrated that PLK1 depletion caused cell cycle arrest at mitosis and induced apoptosis in several cancer cell lines [23]. Therefore, PLK1 is a possible therapeutic target for the treatment of human cancer.

A chemical proteomic method, referred to as activity-based protein profiling (ABPP), that utilizes active site-directed probes to monitor the functional state of enzyme families in complex proteomes has been developed [24–26]. This approach can be contrasted with conventional proteomics, for example, two-dimensional gel electrophoresis (2DE) coupled with protein staining and mass spectrometry (MS), which are restricted to recording variations in protein abundance [26]. To date, activity-based probes (ABPs) that target serine hydrolases [24, 27, 28], cysteine proteases [29–31], serine-threonine phosphatases [32], metalloproteases [33], as well as several metabolic enzymes, including dehydrogenases [25], sugar kinases, and glutathione S-transferases [34, 35] have been successfully generated.

Jessani and colleagues utilized ABPs that target serine hydrolases to identify several secreted and membrane-associated enzyme activities upregulated in invasive breast and melanoma cancer cells, including the secreted protease urokinase, and novel enzymes, like the membrane-associated hydrolase KIAA1363 [36]. More recently, Greenbaum and colleagues utilized ABPs that target cysteine proteases to identify falcipain 1 as a protease that is uniquely active in the invasive merozoite stage of Plasmodium falciparum, the human malaria parasite [37]. To support falcipain 1 as a target for antimalarial drugs, the authors showed that falcipain 1 inhibitors, identified by utilizing ABPP methods, strongly inhibited parasite invasion of host erythrocytes. Collectively, these findings demonstrate that ABPP methods provide a powerful tool for discovering enzymes associated with human disease and chemical inhibitors that target these enzymes by the screening of compounds.

We report here the chemical synthesis and utility of a tetramethylrhodamine-wortmannin conjugate ABP, AX7503. Wortmannin has been reported to be a highly selective inhibitor of PI 3-kinase and PI 3-kinase-related kinases, such as ATM, ATR, and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) [38–40]. The IC_{50} of wortmannin for PI 3-kinase is 4.2 nM [38]. Surprisingly, AX7503 was found to label PLK1 in addition to members of the PI 3-kinase superfamily in complex proteomes. We show for the first time, to our knowledge, that wortmannin and LY294002, compounds previously thought to be highly selective inhibitors of PI 3-kinases, also inhibit PLK1. In addition, we show that wortmannin potently inhibits PLK1 activity in an in vitro kinase assay and inhibits AX7503 labeling of PLK1 when incubated with intact G2/M-arrested cells. Finally, we highlight the utility of AX7503 as a probe for

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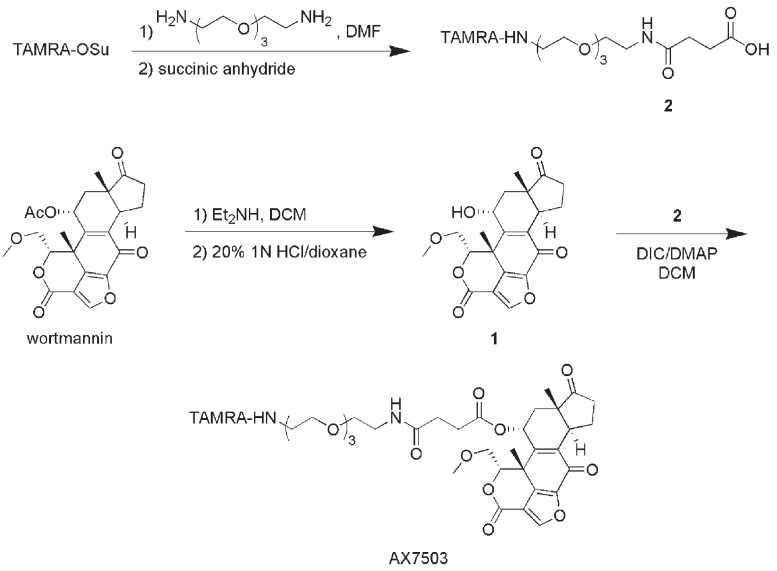

Figure 1. Synthesis of AX7503

monitoring dynamics in the expression of PLK1 in crude cellular extracts.

Results and Discussion

Synthesis of the AX7503 Probe

In order to study the PI 3-kinase superfamily of enzymes and identify other potential target enzymes inhibited by wortmannin in complex proteomes, we synthesized a tetramethylrhodamine (TAMRA)-wortmannin conjugate, AX7503 (Figure 1). The TAMRA tag permits both visualization and purification of proteins modified by AX7503. Briefly, the synthesis of AX7503 started with the rearrangement of wortmannin to 11-O-desacetylwortmannin (compound **1**). Using a modification of the Haefliger synthesis [41], wortmannin was treated with diethylamine (30 equiv.) in dichloromethane overnight to yield crude 11-O-desacetyl-20-(N,N-diethylimino)-20-desoxowortmannin. After removal of solvent, this material was treated with 20% 1 N HCl/dioxane, which resulted in reclosure of the furan ring. Purification with silica gel chromatography yielded compound **1** in 78% yield. Importantly, modification of the 11-hydroxyl functionality of compound **1** through esterification with long-chain acids or conjugation to polymers has been shown to result in analogs with minimal changes in potency against PI 3-kinase when compared to wortmannin [42, 43]. The lack of sensitivity to modification at this position is explained by the crystal structure of the wortmannin-porcine PI3Kγ complex, in which the 11-acetoxy group of wortmannin is directed away from the active site into solvent [38]. Consequently, conjugation of TAMRA through the 11-OH position was expected to result in a potent probe not only for PI 3-kinase, but also for the other wortmannin-reactive members of the PI 3-kinase family. The requisite long-chain TAMRA derivative **2** was synthesized in a two-step, one-pot procedure. Treatment of the succinimidyl active ester of TAMRA with tetraethyleneglycol diamine, followed by the addition of succinic anhydride, resulted in the desired product, which was readily separated from impurities by using C_{18} reverse-phase chromatography. Esterification of the alcohol **1** with the carboxylic acid **2** was accomplished by using DIC/DMAP in DCM to yield AX7503 in 31% isolated yield.

Characterization of AX7503

To test the reactivity of AX7503 with the PI 3-kinase superfamily of enzymes, we incubated soluble fractions of Jurkat cells with this probe at 50 nM for 60 min, after which the fluorescently labeled proteins were separated by SDS-PAGE and visualized in-gel with a flatbed laser-induced fluorescence scanner. As seen in Figure 2A, AX7503 specifically labeled more than five protein bands (I, II, and III). The labeling of these proteins, but not several that may be labeled nonspecifically by AX7503, was blocked when the soluble fractions were preincubated with 0.5 μM wortmannin or 50 μM LY294002 for 10 min prior to treatment with the probe (Figure 2A). When enzymatic activity was abolished by heat denaturation, AX7503 did not label the proteins in bands I, II, and III (data not shown). Affinity isolation of probe-labeled proteins by virtue of the TAMRA tag of AX7503 and mass spectrometric analysis identified the 465 kDa protein (I) as a catalytic subunit of DNA-PK (gi|13654237, 20 peptides matched, 6% sequence coverage), the 110–125 kDa proteins (II) as the catalytic subunits of the PI 3-kinase α (gi|1763626, 23 peptides matched, 22% sequence coverage), the PI 3-kinase β (gi|5453894, 16 peptides matched, 14% sequence coverage), and the PI 3-kinase γ (gi|12620871, 4 peptides matched, 5% sequence coverage). These results confirmed that AX7503 performed as designed to covalently label the PI 3-kinase superfamily. Interestingly, the 68 kDa protein (III) was identified as PLK1 (gi|393017, 5 peptides matched, 13% sequence coverage), which shares no significant sequence identity with the PI 3-kinase superfamily. Western blot analysis with a monoclonal PLK1 antibody confirmed that AX7503-labeled proteins immuprecipitated via the rho-

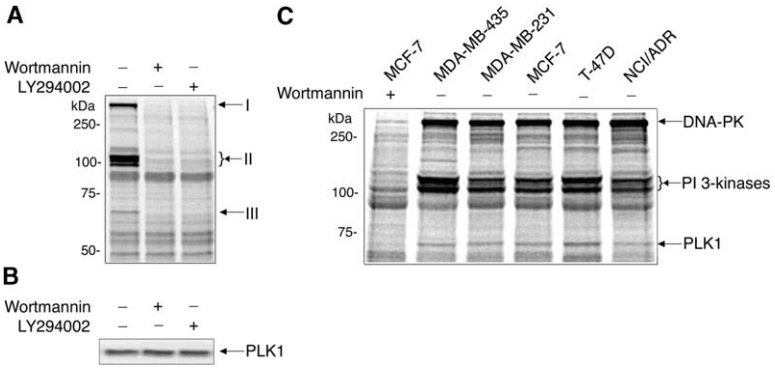

Figure 2. Identification of AX7503-Reactive Proteins

(A) Jurkat cells were grown in RPMI medium 1640 containing 10% fetal calf serum in a humid atmosphere of 5% CO_2 at 37°C, and soluble proteome (1.5 mg/ml) derived from these cells was preincubated with or without wortmannin (0.5 μM) or LY294002 (50 μM) for 10 min and then treated with AX7503 (50 nM) for 60 min. Reactions were quenched with standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, and visualized in-gel with a flatbed laser-induced fluorescence scanner (MiraBio).

(B) The scanned gel from (A) was transferred to nitrocellulose and immunoblotted with anti-PLK1.

(C) Five breast cancer cell lines were grown to 80% confluence in RPMI medium 1640 containing 10% fetal calf serum in a humid atmosphere of 5% CO_2 at 37°C, and soluble proteome (1.5 mg/ml) derived from these cells was preincubated with or without wortmannin (0.5 μM) for 10 min and then treated with AX7503 (50 nM) for 60 min. Reactions were quenched with standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, and visualized in-gel with a flatbed laser-induced fluorescence scanner (MiraBio).

damine tag contained the 68 kDa PLK1 (data not shown). Importantly, PLK1 was not labeled by AX7503 in extracts preincubated with wortmannin and LY294002 (Figure 2A) even though the protein is clearly present (Figure 2B). These results demonstrated that AX7503 was not only an effective probe for the PI 3-kinase superfamily, but for PLK1 as well. These findings emphasize that enzymes with no sequence similarity may have related active site structures and that ABPP can be used to discover such relatedness [25].

Human PLK1 has been shown to be highly expressed in various human cancers. We selected a panel of human breast cancer cell lines for analysis with AX7503. As seen in Figure 2C, AX7503 specifically labeled PLK1 in addition to PI 3-kinases and DNA-PKcs from all five breast cancer cell lines. The labeling of these proteins by AX7503 was potently inhibited when the soluble fractions from MCF-7 and the other cell lines were preincubated with 0.5 μM wortmannin for 10 min prior to treatment with the probe (Figure 2C and data not shown). Also, these cell lines expressed PLK1 at similar levels. These results indicated that AX7503 can be used to label and compare the expression levels of PLK1 from different cell lines and tissues.

To test whether AX7503 is an activity-dependent probe for PLK1, we transfected constructs encoding both wild-type PLK1 and the catalytically inactive mutant PLK1 (K82R) into HEK-293 cells [44]. As seen in Figure 3A, treatment of the soluble fractions of these cells with AX7503 resulted in the intense labeling of PLK1 in wild-type PLK1-transfected cells, but only weak labeling of PLK1 in the mutant PLK1-transfected cells. The weak labeling of PLK1 in the mutant-containing cells was consistent with that seen for control cells transfected with empty vector as well as untransfected cells. Thus, the weak labeling of PLK1 in the mutant-containing cells likely represents endogenous levels of PLK1 in this cell type. Western blot analysis with a monoclonal PLK1 antibody identified comparable levels of overexpression for both wild-type and mutant PLK1 (Figure 3B). These results demonstrated that AX7503 exclusively reacted with the catalytically active form of the enzyme. Since PLK1 activity is regulated by phosphorylation, we treated recombinant PLK1 with purified protein phosphatase 2A (PP2A), as PP2A treatment of PLK1 has been shown to decrease the rate of PLK1-mediated phosphorylation of casein [15]. The dephosphorylated PLK1 was labeled by AX7503 at a similar rate to nondephosphorylated PLK1 (data not shown). These results are consistent with previous reports that the phosphorylation is not required for activity and serve to highlight potential kinetic differences between single turnover and multiple turnover reactions [15].

To determine the potency of wortmannin and LY294002 as inhibitors of PLK1, we quantified competition between AX7503 and either wortmaninn or LY294002 for kinases in Jurkat-soluble fractions. By fitting the dose-response data in Figure 3A, wortmannin's

Figure 3. AX7503 Reacts with PLK1 in an Activity-Dependent Manner

(A) Soluble proteome (1.5 mg/ml) from parental HEK-293 cells and cells transfected with empty vector (Mock), a wild-type PLK1 construct, and the mutant K82R PLK1 construct was treated with AX7503 (50 nM) for 60 min. Reactions were quenched with standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, and visualized in-gel with a flatbed laser-induced fluorescence scanner (MiraBio).

(B) The scanned gel from (A) was transferred to nitrocellulose and immunoblotted with anti-PLK1.

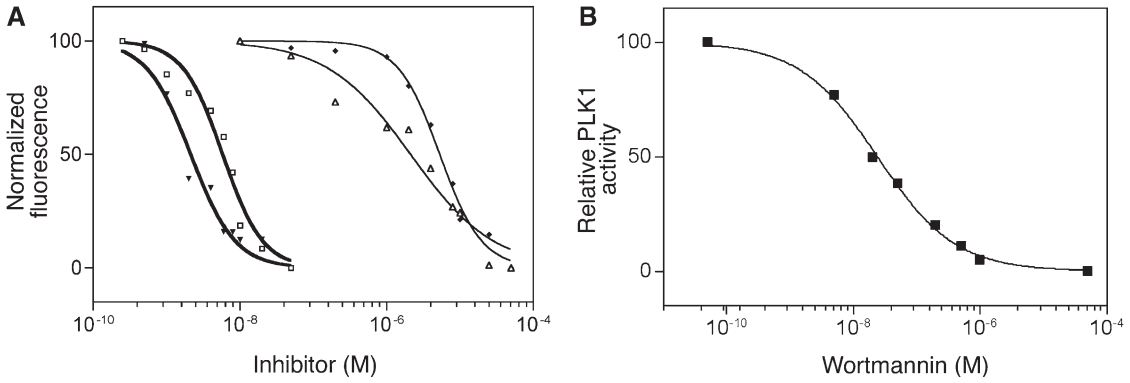

Figure 4. Wortmannin and LY294002 Are Inhibitors of PLK1

(A) Dose-response curves of wortmannin and LY294002 for AX7503 labeling of PLK1 and PI 3-kinases. Jurkat-soluble fractions were treated with AX7503 (50 nM) for 60 min with or without preincubation with various concentrations of wortmannin (0.25–50 nM) or LY294002 (10–50,000 nM) for 10 min. Reactions were quenched with standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, and visualized in-gel with a flatbed laser-induced fluorescence scanner (MiraBio). Fluorescently labeled PLK1 and PI 3-kinases were quantified by measuring integrated band intensities (normalized for volume); control samples (DMSO alone) were considered to have 100% activity, and wortmannin and LY 294002-treated samples were expressed as a percentage of remaining activity. IC_{50} values were determined from dose-response curves by using Prism software (GraphPad). Open square, PLK1-wortmannin; open triangle, PLK1-LY294002; closed triangle, PI 3-kinase-wortmannin; closed diamond, PI 3-kinase-LY294002.

(B) Inhibition of PLK1 by wortmannin in an in vitro kinase assay. A nonradioactive PLK1 assay was used (Clyclex Polo-like kinase 1 Assay/Inhibitor Screening Kit). The kit uses recombinant Protein-X as a substrate containing threonine residues that can be efficiently phosphorylated by PLK1. The amount of phosphorylated substrate is measured by using an anti-phospho-threonine polyclonal antibody (PPT-07), followed by binding with horseradish peroxidase-conjugated anti-rabbit IgG, which then catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution. The blue solution changes to yellow after the addition of stopping solution. The color is quantitated by spectrophotometry and reflects the relative amount of PLK1 activity. Briefly, 50 μM ATP, wortmannin at indicated concentrations, and 3 mU GST-tagged PLK1 in kinase buffer were added to a plate precoated with recombinant Protein-X in a final volume of 100 μl, and the plate was incubated for 30 min at 30°C. ATP minus control and no enzyme control were included for every experiment.

IC_{50} value for inhibiting PLK1 reactivity was determined to be 5.8 nM, whereas LY294002's IC_{50} value was determined to be 2.1 μM. Wortmannin and LY294002 inhibited PI 3-kinases with similar potency to their inhibition of PLK1; their IC_{50} values for PI 3-kinases were 2.1 nM and 5.3 μM, respectively (Figure 4A).

To obtain independent evidence for the ability of wortmannin to inhibit PLK1, we performed an in vitro kinase assay for the ability of pure PLK1 to phosphorylate a recombinant substrate. As seen in Figure 4B, PLK1 activity was inhibited by wortmannin with an IC_{50} value of 24 nM, consistent with the results obtained from Figure 4A. Using the same assay, we also found that AX7503 inhibited PLK1 with similar potency to wortmannin itself (data not shown). Further, LY294002 inhibited PLK1 in the in vitro kinase assay with similar potency to its inhibition of AX7503 labeling of PLK1 (data not shown).

In Vivo Inhibition of PLK1 by Wortmannin

The level and activity of PLK are regulated during the cell cycle. In mammalian cells, PLK1 protein levels are low in G1 and S phase but start to rise in G2 and reach maximal in mitosis [45, 46]. To test whether AX7503 could be used to observe cell cycle-dependent changes in PLK1 protein levels in crude cellular extracts, we chemically synchronized Jurkat cells at either G1/S or G2/M. As seen in Figure 5A, AX7503 labeled PLK1 strongly in the soluble fraction of the G2/M-arrested cells, but only labeled PLK1 weakly in the G1/S-arrested cells. In contrast to PLK1, PI 3-kinases and DNA-PKcs expression were not changed in G1/S- or G2/M-arrested Jurkat cells (Figure 5A). Western blot analysis confirmed the upregulation and downregulation of PLK1 expression in G2/M- and G1/S-arrested cells, respectively (Figure 5B). These results indicated that AX7503 could be used to monitor the changes in PLK1 expression during the cell cycle.

Since wortmannin is widely used to experimentally inhibit PI 3-kinases, it was of interest to determine the amount of PLK1 inhibition that results from treating live cells with wortmannin. Intact G2/M-arrested Jurkat cells were treated with a range of concentrations of wortmannin for 30 min. After 30 min, cells were collected, washed, and lysed. AX7503 treatment of these lysates could then be used to assay for the inhibition of its targets by wortmannin treatment of whole cells. As shown in Figure 5C, treatment of the G2/M-arrested Jurkat cells with wortmannin led to a decrease in the activity of PI 3-kinases and PLK1. While wortmannin inhibited PI 3-kinases more potently than PLK1, wortmannin significantly inhibited PLK1 at concentrations above 25 nM (Figure 5C). For example, a 37% decrease in PLK1 activity was observed when the G2/M-arrested cells were treated with wortmannin at 100 nM. Western blot analysis with a monoclonal PLK1 antibody confirmed that the expression of PLK1 did not change upon treating cells with wortmannin (data not shown). These results indicated that, at the wortmannin concentrations used to inhibit PI 3-kinases in cell-based

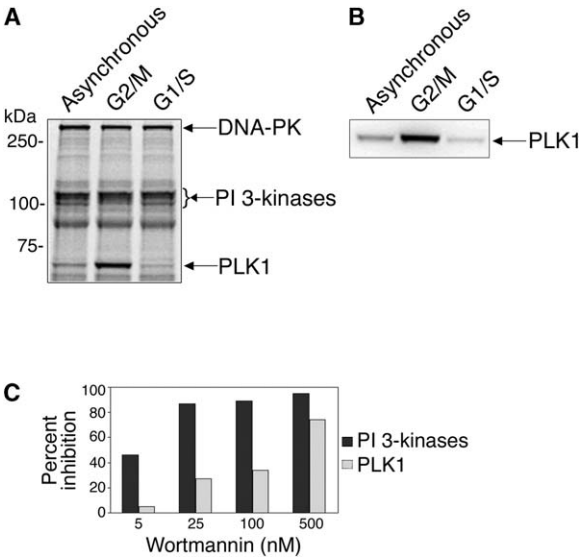

Figure 5. In Vivo Inhibition of PLK1 by Wortmannin

(A) AX7503 detects the upregulation of PLK1 expression in G2/M-arrested Jurkat T cells. Jurkat cells were treated with 3 mM hydroxyurea for 16 hr (G1/S arrest), 10 μM nocodzole for 15 hr (G2/M arrest), or control DMSO, and soluble proteome (1.5 mg/ml) derived from these cells was treated with AX7503 (50 nM) for 60 min. Reactions were quenched with standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, and visualized in-gel with a flatbed laser-induced fluorescence scanner (MiraBio).

(B) The scanned gel from (A) was transferred to nitrocellulose and immunoblotted with anti-PLK1.

(C) Jurkat cells were first treated with 10 μM nocodazole for 15 hr (G2/M arrest). The intact cells were then treated with indicated concentrations of wortmannin or control DMSO for 30 min. Cells were collected, washed, and lysed. Soluble proteome (1.5 mg/ml) derived from these cells was treated with AX7503 (50 nM) for 60 min. Reactions were quenched with standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, and visualized in-gel with a flatbed laser-induced fluorescence scanner (MiraBio).

assays (50–500 nM), PLK1 inhibition could be physiologically relevant. In addition, wortmannin is also used to inhibit PI 3-kinase-related kinases such as ATM at much higher concentrations (as high as 5 μM) (see, for example [23]). Because wortmannin inhibits PLK1 in intact G2/M-arrested cells and PLK1 is essential for mitotic spindle assembly [6, 10], these results also indicate that wortmannin should inhibit mitotic progression through inhibition of PLK1.

AX7503 Detects Changes in PLK1 Expression in Cells Treated with Drugs

Paclitaxel poisons microtubules by disrupting the dynamic equilibrium between soluble tubulin dimers and their polymerized form and is a potent inhibitor of chromosome replication. Thus, it prevents normal cell division and arrests the cell cycle at M phase [47]. Since PLK1 expression is high in the mitotic phase, PLK1 expression should be upregulated upon paclitaxel treatment. To test this hypothesis, we treated Jurkat cells with paclitaxel and, as expected, the AX7503-labeled PLK1 was more abundant in the soluble fraction of treated than untreated cells (Figure 6A). Western blot analysis confirmed the upregulation of PLK1 expression in the paclitaxel-treated cells (data not shown). In addition to poisoning microtubules, paclitaxel also induces apoptosis. We therefore looked for PLK1 changes upon treatment with other inducers of apoptosis, staurosporine and camptothecin (CPT). In contrast to paclitaxel, staurosporine and CPT had little effect or weakly decreased PLK1 expression levels (Figure 6A). These results suggest that the differences in drug-dependent PLK1 expression were caused by the efficiency of the drugs to trigger the mitotic checkpoint. The mitotic spindle-destroying drug paclitaxel might induce apoptosis through genes that regulate and control the spindle assembly checkpoint of mitosis.

Paclitaxel is one of the major drugs used in the treatment of ovarian and breast cancer. In order to determine whether paclitaxel also causes the upregulation of PLK1 in cancer cells, we treated breast cancer line MDA-MB-435 cells with paclitaxel as described in the Experimental Procedures. As shown in Figure 6B, paclitaxel strongly induced the upregulation of PLK1 expression in MDA-MB-435 cells. These results indicate that PLK1 expression, as determined by AX7503 labeling, could serve as a biomarker for paclitaxel efficacy in breast cancer treatment.

To test the ability of AX7503 to detect the upregulation of PLK1 expression in cells treated with a drug that drives cells into a state resembling mitosis, we treated Jurkat cells with calyculin A, a serine/threonine phosphatase PP1 and PP2A inhibitor [48]. As shown in Figure 6C, AX7503 labeled more PLK1 in Jurkat cells treated with calyculin A compared with control Jurkat cells not treated with calyculin A. PLK1 expression, as quantified by integrated fluorescent band intensity, was 2.3-fold higher in calyculin A-treated Jurkat cells relative to control Jurkat cells (Figure 6D). These results indicate that PLK1 expression was dramatically increased in calyculin A-stimulated cells and AX7503 could detect these changes in PLK1 expression in crude cellular extracts. These results are consistent with previous reports that calyculin A causes cells to arrest in G2/M. The increased portion of cells in G2/M in the calyculin A-treated cells presumably leads to increased levels of PLK1. Collectively, these results highlight AX7503's ability to monitor the dynamics in PLK1 expression during the cell cycle.

Due to its integrated fluorescent moiety and direct 1:1 labeling of its target, AX7503 may have utility in the quantification of PLK1, which, as noted earlier, may be a useful biomarker in oncology. Importantly, although PLK1 is a possible target for treatment of cancer, very few PLK1 inhibitors have been reported. One such inhibitor is scytonemin, which inhibits PLK1, as well as other cell cycle regulatory kinases like Myt1, checkpoint kinase 1, cyclin-dependent kinase 1/cyclin B, and protein kinase Cβ2 in the low micromolar range [49]. Therefore, wortmannin may provide a valuable lead for the development of PLK1 inhibitors.

The evidence that, in contrast to anti-PLK1 antibodies, AX7503 is an activity-based probe for PLK1 follows. First, the labeling of PLK1 by AX7503 is blocked by the small-molecule inhibitors wortmannin and LY294002 and by heat denaturation. Second, AX7503

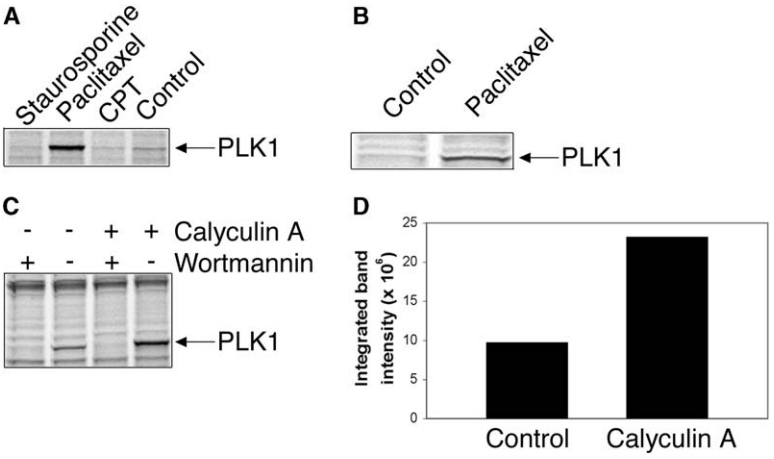

Figure 6. AX7503 Detects Changes in PLK1 Expression in Cells Treated with Drugs

(A) Jurkat cells were treated with 2 μM paclitaxel for 20 hr, 1 μM staurosporine for 2 hr, 3 μM capmtothecin for 20 hr, or control DMSO, and soluble proteome (1.5 mg/ml) derived from these cells was treated with AX7503 (50 nM) for 60 min. Reactions were quenched with standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, and visualized in-gel with a flatbed laser-induced fluorescence scanner (MiraBio).

(B) MDA-MB-435 cells were treated with 2 μM paclitaxel for 20 hr or control DMSO, and soluble proteome derived from these cells was treated with AX7503 (50 nM) for 60 min. Reactions were quenched with standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, and visualized in-gel with a flatbed laser-induced fluorescence scanner (MiraBio).

(C) Jurkat cells were treated with 25 nM calyculin A or control DMSO for 30 min, and soluble proteome derived from these cells was treated with AX7503 (50 nM) for 60 min. Reactions were quenched with standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, and visualized in-gel with a flatbed laser-induced fluorescence scanner (MiraBio).

(D) Fluorescently labeled PLK1 was quantified by measuring integrated band intensities (normalized for volume).

exclusively reacts with the catalytically active form of PLK1. Furthermore, AX7503 can measure the increased or decreased total PLK1 activity resulting from changes in expression in crude extracts. Finally, AX7503 inhibits PLK1 activity in vitro and covalently labels PLK1, further supporting the notion that this tagged inhibitor reacts with PLK1 in an activity-dependent manner.

Significance

We report the synthesis and initial characterization of AX7503, a wortmannin-based ABP that is able to dynamically read out activity changes of a limited set of kinases within crude biological mixtures. We have provided examples of activity changes deriving from small-molecule inhibitor treatment of extracts or from changes in protein expression in cells treated with drugs or synchronized at stages of the cell cycle. Unexpectedly, we discovered that wortmannin potently inhibits PLK1 in addition to the PI 3-kinase superfamily. Wortmannin's potency for PLK1 is about 1000-fold higher than that of a previously published PLK inhibitor, scytonemin. Importantly, we demonstrated that wortmannin potently inhibits PLK1 activity in vitro and also inhibits PLK1 when added to live cells. At the concentrations of wortmannin commonly used to inhibit PI 3-kinases, PLK1 is also significantly inhibited. Wortmannin may be a valuable lead for the development of PLK1 inhibitors for cancer treatment, and AX7503 provides a unique secondary screening method to analyze specificity of PLK1 inhibitors, including wortmannin or LY294002 derivatives.

Experimental Procedures

Synthesis of AX7503

All solvents and reagents were obtained from the Aldrich Chemical Company (Milwaukee, WI) unless otherwise indicated. The mixed succinimidyl ester of 5-(and 6-)carboxytetramethylrhodamine (TAMRA-OSu) was obtained from Molecular Probes (Eugene, OR), and wortmannin was obtained from A.G. Scientific, Inc. (San Diego, CA). Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker 400 MHz NMR spectrometer in deuterated dimethylsulfoxide (d_6-DMSO) using the residual solvent peak as the internal standard. LC/(ES)MS analysis was performed on an Agilent 1100 Series LC/MSD with ChemStation software. Preparative HPLC was carried out on a C_{18} reverse-phase column (Polaris, 5 μ column, 150 mm × 21 mm; Varian; Torrance, CA) using a binary system of water and acetonitrile with 0.1% trifluoroacetic acid as a modifier. Analytical LC/MS was carried out on a C_{18} reverse-phase column (Polaris, 5 μ column, 50 × 4.6 mm; Varian; Torrance, CA) using a binary system of water and acetonitrile with 0.1% trifluoroacetic acid as a modifier. The purity of HPLC fractions was determined by analyzing the LC/MS trace at both 220 nm and 550 nm.

N-(2-{2-[2-(2-TAMRA-Amino-Ethoxy)-Ethoxy]-Ethoxy}-Ethyl)-Succinamic Acid [2]

To a solution of tetraethyleneglycol diamine (10.5 mg, 54.6 μmol) in DMF (280 μl) was added TAMRA-OSu (14.4 mg, 27.3 μmol) under argon. The solution was allowed to stir at room temperature for 1 hr. Succinic anhydride (5.3 mg, 53.0 μmol) then was added to the solution, followed by triethylamine (6.9 mg, 68.3 μmol). The reaction solution was flushed with argon and allowed to stir at room temperature for 23 hr. The reaction mixture was then purified by reverse-phase chromatography. The fractions containing the product in >95% purity by HPLC were collected and lyophilized overnight, affording compound **2** as a purple glass (11.9 mg, 62%). ^{1}H NMR δ: 2.30 (t, 2H, *J* = 6.8 Hz), 2.39 (t, 2H, *J* = 6.8 Hz), 3.11–3.30 (m, 12H), 3.45–3.65 (m, 16H), 6.57 (b, 1H), 6.80–7.10 (b, 5H), 7.55 (b, 1H), 7.91 (t, 1H, *J* = 5.2 Hz), 8.30 (d, 1H, *J* = 8.0 Hz), 8.67 (b, 1H), 8.97 (t, 1H, *J* = 4.4 Hz); ESMS: 705.0 $[M + H]^+$, 353.0 $[M + 2H]^{2+}$; MALDI-FTMS (DHB): m/z 705.3121 ($C_{37}H_{44}N_4O_{10}$ + H requires 705.3136).

AX7503

To a solution of compound **2** (8 mg, 11 μmol) and 11-*O*-desacetylwortmannin (**1**, 4.4 mg, 11.4 μmol) in CH_2Cl_2 (2.0 mL) was added DMAP (2 mg, 16 μmol), followed by DIC (6.75 μL, 43 μmol). The reaction was stirred at ambient temperature for 16 hr. The solvent was removed under vacuum, and the resulting solid was dissolved in a minimal amount of DMSO and purified by reverse-phase chromatography. The fractions containing the product in >95% HPLC purity were collected, and solvent was removed under vacuum, affording AX7503 as a reddish glass (3.8 mg, 31%). ^{1}H NMR δ: 1.23 (s, 3H), 1.56 (dd, 2H, *J* = 10.8 Hz), 1.63 (s, 3H), 1.90–2.03 (m, 3H), 2.17 (m, 2H), 2.26–2.47 (m, 4H), 3.06 (s, 3H), 3.15–3.28 (m, 12H), 3.40–3.62 (m, 16H), 4.13 (dd, 2H, *J*= 3.4 Hz), 5.12 (dd, 1H, *J* = 1.6 Hz), 5.94 (m, 1H), 6.55 (b, 1H), 6.87–7.20 (b, 5H), 7.54 (b, 1H), 7.98 (t, 1H, *J* = 5.2 Hz), 8.29 (d, 1H, *J* = 6.8 Hz), 8.67 (b, 1H), 8.94 (s,

1H), 8.95 (b, 1H); ESMS: 1073.4 $[M + H]^+$; MALDI-FTMS (DHB): m/z 1073.4375 ($C_{58}H_{64}N_4O_{16}$ + H requires 1073.4396).

Cell Culture, Chemical Cell Cycle Synchronization, and Inhibitor Treatment

Jurkat cells were grown in RPMI medium 1640 containing 10% fetal calf serum (GIBCO-BRL, Invitrogen Corp.) in a humid atmosphere of 5% CO_2 at 37°C. The breast cancer lines MDA-MB-435, MDA-MB-231, MCF-7, T-47D, and NCI/ADR were obtained from the National Cancer Institute's Developmental Therapeutics Program. All breast cancer cell lines were grown to 80% confluence in RPMI medium 1640 containing 10% fetal calf serum. For paclitaxel treatment, MDA-MB-435 cells were grown to 80% confluence in RPMI medium 1640 containing 10% fetal calf serum and were then treated with paclitaxel. For drug-induced cell synchronization, Jurkat cells were treated with 3 mM hydroxyurea for 16 hr (G1/S arrest) and 10 μM nocodazole for 15 hr (G2/M arrest) [3]. For in vivo inhibition of PLK1 by wortmannin, Jurkat cells were first treated with 10 μM nocodzole for 15 hr (G2/M arrest) and then treated with wortmannin at the concentration indicated in the figure legends for 30 min. For inhibitor treatment, Jurkat cells were incubated in media containing calyculin A (25 nM), staurosporine (1 μM), paclitaxel (2 μM), or camptothecin (3 μM) for the time indicated in the figure legends [50–53]. Stock solutions of inhibitors were prepared in dimethyl sulfoxide. Control incubations included the same amount of dimethyl sulfoxide as the stock solutions.

Preparation of Cell Lysates

Cells were harvested by centrifugation and washed twice with cold phosphate-buffered saline (pH 7.4). Cell pellets were briefly sonicated and Dounce homogenized in 50 mM HEPES (pH 7.4) containing 400 mM β-glycerol phosphate, 100 mM sodium pyrophosphate, and 500 mM sodium sulfate (buffer 1). Cell extracts were centrifuged at 100,000 × g to provide soluble cellular proteome fractions (supernatant).

Labeling of Protein Samples with AX7503

Soluble fractions were adjusted to a final protein concentration of 1.5 mg/ml in buffer 1 and treated with 50 nM AX7503 for 1 hr at room temperature. Control reactions consisting of the soluble fractions were preincubated with 0.5 μM wortmannin or 50 μM LY294002 for 10 min prior to the addition of AX7503. Protein bands that reacted with AX7503 in both preincubated and unpreincubated samples with wortmannin or LY294002 were defined as nonspecific labeling. Reactions were quenched with one volume of standard 2× SDS/PAGE loading buffer (reducing), separated by SDS/PAGE, and visualized in-gel with a Hitachi FMBio IIe flatbed fluorescence scanner (MiraiBio) as described [28, 36]. Fluorescently labeled PLK1 and PI 3-kinases were quantified by measuring integrated band intensities (normalized for volume). IC_{50} values were determined from dose-response curves with Prism software (GraphPad).

Isolation and Identification of AX7503-Labeled Proteins

AX7503-labeled proteins were isolated by using an immunoaffinity-based purification procedure [54]. The enriched TAMRA-wortmannin-labeled proteins were separated by using SDS-PAGE, and the protein bands were excised and digested with trypsin. The resulting peptides were analyzed by high-throughput microcapillary reverse-phase chromatography (100 μm capillary, 10 cm, C_{18}) in parallel with ion trap mass spectrometry (LCQ Deca XP, Thermofinnigan, San Jose, CA). Peptides were identified by using the Mascot search algorithm and by de novo sequencing.

Polo-like Kinase 1 Activity Assay

An in vitro PLK1 assay in the presence and absence of various inhibitors was performed by using the Clyclex Polo-like kinase 1 Assay/Inhibitor Screening Kit (CycLex Co., Ltd., Japan) according to the manufacturer's protocol, except that purified GST-tagged PLK1 was diluted 60-fold into kinase buffer.

Western Blot Analysis

Quenched AX7503 reactions were run on SDS-PAGE (20 μg/lane). Proteins were then transferred by electroblotting onto nitrocellulose membranes (Protran). Western blot analysis with the anti-Polo-like kinase mouse monoclonal antibody (Zymed, CA) was conducted as described previously.

Expression of PLK1 in HEK-293 Cells

The HA-tagged PLK constructs, wild-type and the catalytically inactive mutant K82R, and empty vector were transiently transfected into HEK-293 cells by using methods described previously [44].

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