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Article

Inhibition of Polo-like Kinase 1 by Blocking Polo-Box Domain-Dependent Protein-Protein Interactions

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SUMMARY

The serine/threonine kinase Polo-like kinase 1 (Plk1) is overexpressed in many types of human cancers, and has been implicated as an adverse prognostic marker for cancer patients. Plk1 localizes to its intracellular anchoring sites via its polo-box domain (PBD). Here we show that Plk1 can be inhibited by small molecules which interfere with its intracellular localization by inhibiting the function of the PBD. We report the natural product thymoquinone and, especially, the synthetic thymoquinone derivative Poloxin as inhibitors of the Plk1 PBD. Both compounds inhibit the function of the Plk1 PBD in vitro, and cause Plk1 mislocalization, chromosome congression defects, mitotic arrest, and apoptosis in HeLa cells. Our data validate the Plk1 PBD as an anticancer target and provide a rationale for developing thymoquinone derivatives as anticancer drugs.

INTRODUCTION

The serine/threonine kinase Polo-like kinase 1 (Plk1) is a regulator of multiple stages of mitotic progression. It is overexpressed in many types of human cancers, and has been implicated as an adverse prognostic marker for cancer patients (Barr et al., 2004; McInnes et al., 2005; Strebhardt and Ullrich, 2006). Its inhibition in cancer cells by small interfering RNA (Liu and Erikson, 2003; Spankuch-Schmitt et al., 2002a), a Plk1-derived peptide (Yuan et al., 2002), antisense oligonucleotides (Spankuch-Schmitt et al., 2002b), and small-molecule inhibitors of its catalytic activity (Gumireddy et al., 2005; Lansing et al., 2007; Lenart et al., 2007; Liu et al., 2005; McInnes et al., 2006; Peters et al., 2006; Santamaria et al., 2007; Steegmaier et al., 2007; Stevenson et al., 2002) has been uniformly demonstrated to induce mitotic arrest and apoptosis of cancer cells in vitro and in vivo. Inhibition of Plk1 by small-molecule inhibitors of its catalytic activity is currently being explored in clinical settings (Gumireddy et al., 2005; Steegmaier et al., 2007). Plk1 localizes to its intracellular anchoring sites via its carboxy-terminal portion containing the polo-box domain (PBD). The PBD consists of two conserved domains referred to as polo boxes, each of which exhibits folds based on a six-stranded β sheet and an α helix, which associate to form a 12-stranded β sandwich domain. Phosphoserine/phosphothreonine-containing peptides comprising an S-(pT/pS)-(P/X) motif (Elia et al., 2003a) bind along a positively charged cleft between the two polo boxes (Cheng et al., 2003; Elia et al., 2003b). Because the PBD is unique to the family of Plks and is essential for Plk functions, the PBD is ideally suited to the exploration of the feasibility of inhibiting a serine/threonine kinase by interfering with its intracellular localization, rather than targeting the conserved ATP binding site. Moreover, small molecules targeting the PBD should be useful research tools to decipher the biological role of the Plk1 PBD in mammalian cells. Here we report our discovery that the natural product thymoquinone and its synthetic derivative Poloxin inhibit the functions of the Plk1 PBD in vitro and in HeLa cells. To our knowledge, this represents the first report of nonpeptidic inhibitors of the Plk1 PBD.

RESULTS

We have developed a fluorescence polarization assay based on binding of the Plk1 PBD to a fluorophore-labeled peptide comprising its optimal recognition motif (Elia et al., 2003a). Screening of diverse chemical libraries consisting of 22,461 small molecules for compounds which could interfere with the function of the Plk1 PBD led to the identification of Poloxin (**1**) (*polo*-box domain *in*hibitor) (apparent IC_{50} of 4.8 ± 1.3 μM) (Figures 1A and 1B; see Table S1 in the Supplemental Data available with this article online). Poloxin's IC_{50} values against the PBDs of Plk2 and Plk3 (Elia et al., 2003b) as the most stringent specificity controls were approximately 4-fold and 11-fold higher, respectively (apparent IC_{50}: Plk2 PBD: 18.7 ± 1.8 μM; Plk3 PBD: 53.9 ± 8.5 μM) in analogous fluorescence polarization assays. Due to its diversity, the screening library did not contain analogs of Poloxin, from which preliminary structure-activity relationships might have been inferred. Poloxin's core structure is represented by the natural product thymoquinone (**2**) (Figure 1A). Thymoquinone is the bioactive constituent of the volatile oil of black seed (*Nigella sativa*), and is well known for its anti-inflammatory and anti-oxidant activities (Gali-Muhtasib et al., 2006). Moreover, numerous studies have demonstrated thymoquinone's potent antineoplastic activity, which seems to be specific for cancer

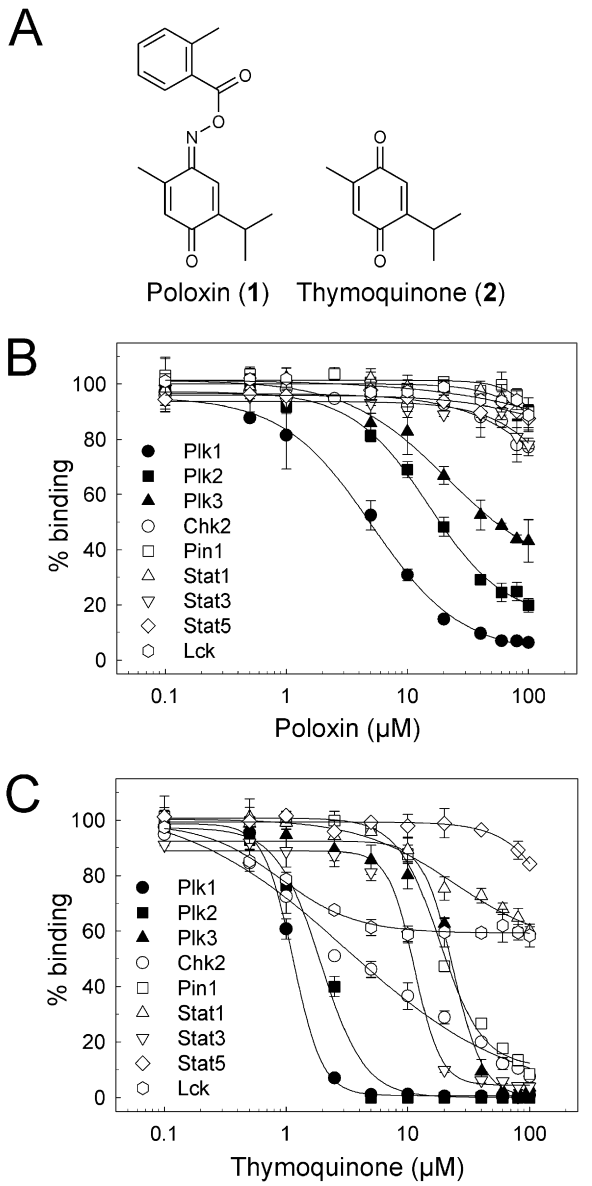

Figure 1. Poloxin and Thymoquinone Inhibit the Plk1 PBD

(A) Chemical structures of Poloxin (**1**) and thymoquinone (**2**).

(B) Effect of Poloxin on binding of fluorescein-labeled phosphopeptides to their respective protein domains assayed by fluorescence polarization. Error bars represent standard deviations (SD).

(C) Effect of thymoquinone on binding of fluorescein-labeled phosphopeptides to their respective protein domains assayed by fluorescence polarization. Error bars represent SD.

cells (Gali-Muhtasib et al., 2006; Kaseb et al., 2007). However, direct molecular targets that could explain its antineoplastic activity have not been reported to date. We found that thymoquinone inhibited the function of the Plk1 PBD even more potently than Poloxin (apparent IC_{50}: 1.14 ± 0.04 μM), but displayed a less desirable specificity profile in that it also affected other subtypes of phosphothreonine/phosphoserine binding domains (Chk2 FHA domain and Pin1 WW domain) and the phosphotyrosine binding Src homology 2 (SH2) domain of STAT3 (Figure 1C; Table S1). Thus, our data suggest that the Plk1 PBD represents a direct molecular target of thymoquinone that could explain its antineoplastic activity.

Inhibition of the Plk1 PBD by both Poloxin and thymoquinone was time dependent, opening the possibility that covalent protein modification may in part contribute to the inhibitor-protein interaction (Figures S1A and S1B). Supporting evidence for covalent modification of Plk1 by Poloxin could arise from experimental approaches which involve incubation of the protein with Poloxin, subsequent tryptic/chymotryptic digest, and identification of peptides by mass spectrometry. Attempts to identify a nucleophilic amino acid modified by Poloxin, however, remained inconclusive. It is conceivable that an amino acid which might have been modified by Poloxin was part of the protein sequence which was not detected in the mass spectrometric analysis; alternatively, protein modification via Michael addition of a nucleophilic amino acid side chain to one of the activated double bonds of Poloxin, or condensation of Poloxin's carbonyl group with a lysine side chain, could have been lost during sample preparation due to the reversible nature of these chemical reactions. Structural data would be required to clarify the protein-inhibitor interactions.

Due to the fundamental role of Plk1 in various stages of mitosis, inhibition of Plk1 by any interfering agent is expected to increase the mitotic index. Mitotic arrest was furthermore observed in tumor cells in which mislocalization of endogenous Plk1 was induced by overexpression of the PBD (Fink et al., 2007; Hanisch et al., 2006; Jiang et al., 2006; Figures S2A and S2B). Consistent with this view, both Poloxin and thymoquinone increased the mitotic index of HeLa cells synchronized by G1/S arrest 14 hr after the release into media containing the test compounds in a dose-dependent manner, as detected by visual inspection of cells under the microscope (Figure 2A, white bars). Most HeLa cells treated with DMSO only as control reached mitosis 9–10 hr after release from aphidicolin-induced G1/S arrest (data not shown). Protein levels of Plk1 are known to increase through G2 phase, peak in M phase, and sharply decrease on exit from mitosis (Barr et al., 2004; Strebhardt and Ullrich, 2006). In order to rule out that the increase in the mitotic index observed 14 hr after release from G1/S arrest was caused by a delay in progression through S phase, in which Plk1 levels are low, cells were released into inhibitor-free media for the first 7 hr, and treated with media containing the test compounds for the next 7 hr (Figure 2A, black bars). Mitotic indices were not decreased under these experimental conditions, arguing against the possibility of off-target effects in S phase as the cause for the mitotic arrest in HeLa cells. Rather, we observed a slight increase in the mitotic indices when the compounds were added 7 hr after G1/S release, possibly because the later addition of the compounds limited the potential for their degradation or inactivation by the components of the tissue culture media. To ensure that all cells, including those entering mitosis earlier than 7 hr after G1/S release, were exposed to the test compounds before mitotic entry, and that the cells were continuously exposed to approximately equal concentrations of intact test compounds, the inhibitors were added at the time of G1/S release, followed by an exchange of the tissue culture media with media containing fresh compounds 7 hr later. Analysis of mitotic indices was performed 14 hr after G1/S release (Figure 2A, gray bars).

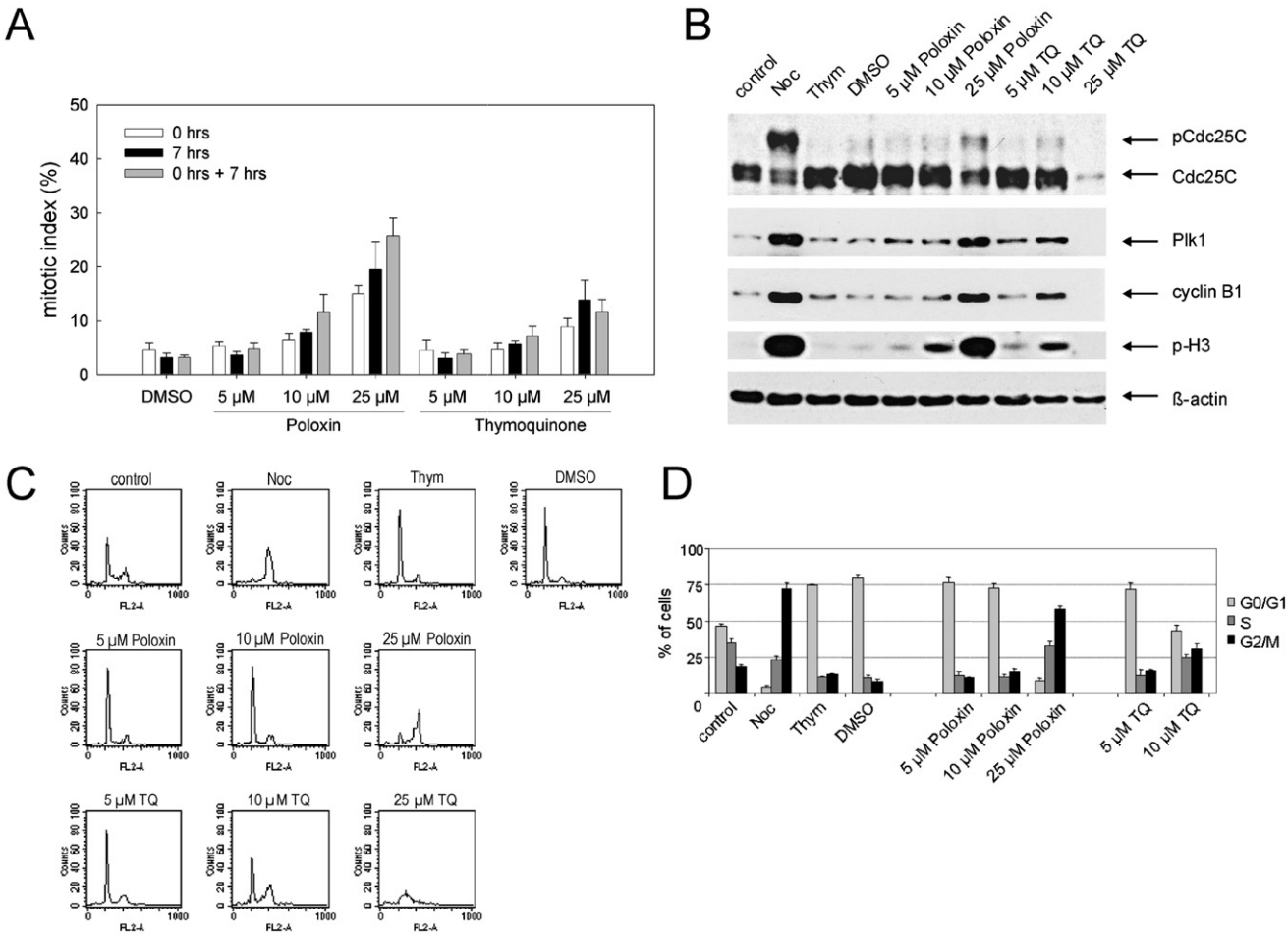

Figure 2. Poloxin and Thymoquinone Induce Mitotic Arrest in HeLa Cells

(A) Mitotic indices of HeLa cells 14 hr after release from G1/S block. Cells were released into medium containing the test compounds at the time of the release (0 hr), 7 hr after release (7 hr), or both (0 hr + 7 hr). The number of mitotic cells within a population of 200–300 cells was counted (n = 3). Error bars represent SD.

(B) HeLa cells were synchronized by thymidine block and released into medium containing Poloxin or thymoquinone (TQ) at the indicated concentrations as described in Experimental Procedures. Unsynchronized cells (control), cells synchronized with nocodazole (Noc) or thymidine (Thym), or cells released from thymidine block in the medium containing only DMSO (DMSO) were analyzed for comparison.

(C) Analysis of cell-cycle distribution by fluorescence-activated cell sorting. Cells were treated as in (B). A representative example is shown.

(D) Quantification of the cell-cycle analysis as shown in (C) (n = 3). Quantification of the cell-cycle profile in the presence of 25 μM TQ was unfeasible due to toxic side effects. Error bars represent SD.

The induction of mitotic arrest by both compounds was confirmed in western blot analysis demonstrating a dose-dependent increase of hyperphosphorylated Cdc25C, a characteristic feature of mitotic cells. Furthermore, we noticed an increase in the concentration of the mitotic markers Plk1, phosphohistone H3, and cyclin B1 (Figure 2B). Mitotic arrest was confirmed by fluorescence-activated cell sorting (FACS), which revealed an increase of cells in G2/M phase and a concomitant decrease in the G0/G1 population for both compounds (Figures 2C and 2D). Thus, even though thymoquinone was about four to five times more active than Poloxin in vitro, the compounds' efficacy to induce mitotic arrest was comparable at a concentration of 10 μM (Figure 2A), possibly because thymoquinone's less selective mode of action decreased the effective concentration available for inhibiting the Plk1 PBD. At 25 μM, thymoquinone displayed toxic side effects, especially under the conditions used for the western blot and FACS analysis (Figures 2B–2D), preventing the quantitative comparison of the compounds' activities.

Mislocalization of Plk1 by overexpression of the Plk1 PBD in HeLa cells had been reported to allow for bipolar spindle formation with properly separated centrosomes. However, most PBD-overexpressing cells were arrested in a prometaphase state due to chromosome congression defects, and clear metaphase plates were rarely observed (Hanisch et al., 2006). Consistent with the literature data, overexpression of the Plk1 PBD led to a strong increase in cells arrested in prometaphase (Figure S2C). Among the cell population arrested in metaphase, the majority displayed chromosome congression defects (Figures S2C and S2D). In contrast, overexpression of an inactive PBD triple mutant had no significant effect on the mitotic index and the distribution of mitotic phases (Figure S2). Consistent with the

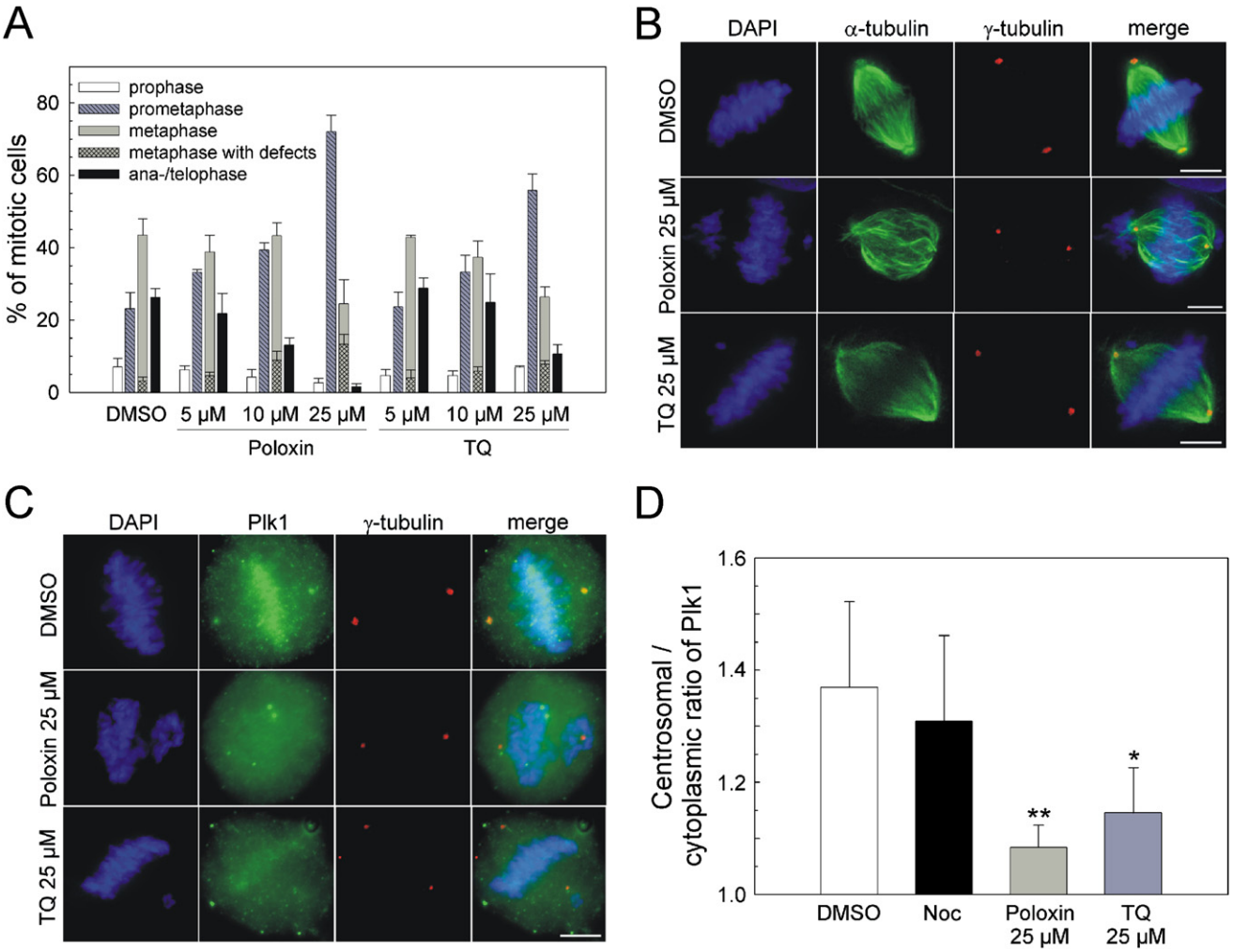

Figure 3. Poloxin and Thymoquinone Induce Chromosome Congression Defects and Mislocalization of Plk1 in HeLa Cells

(A) Distribution of mitotic phases and chromosome congression defects within the total metaphase population in the presence of Poloxin and thymoquinone. Populations of approximately 50 mitotic cells were analyzed (n = 3). Error bars represent SD.

(B) Poloxin and thymoquinone (TQ) induce chromosome congression defects in HeLa cells. Scale bars represent 5 μm.

(C) Poloxin and thymoquinone (TQ) interfere with localization of Plk1 to the centrosomes and kinetochores in metaphase. The scale bar represents 5 μm.

(D) Quantitative analysis of the effects of Poloxin, thymoquinone, and nocodazole (Noc) on centrosomal Plk1 localization in prometaphase and metaphase (Poloxin and thymoquinone) or prometaphase only (nocodazole) (n = 20). *$p < 10^{-5}$ compared to DMSO. **$p < 10^{-7}$ compared to DMSO. p values were calculated using Student's t test (unpaired, one-sided). Error bars represent SD.

idea that the inhibitors induce mislocalization of Plk1 by interfering with the function of the PBD, we observed a dose-dependent increase of cells arrested in prometaphase (Figure 3A) in the presence of Poloxin and thymoquinone. Among the cells that were arrested in a metaphase-like state, we noticed a dose-dependent increase of cells in which single or multiple chromosomes had failed to congress to the metaphase plate (Figures 3A and 3B). The percentage of metaphase cells with chromosome congression defects increased from 7% in the DMSO control to 12%, 21%, and 54% in the presence of 5 μM, 10 μM, and 25 μM Poloxin, respectively. A lesser increase of metaphase cells with chromosome congression defects was observed for thymoquinone (9%, 16%, and 30% in the presence of 5 μM, 10 μM, and 25 μM thymoquinone), indicative of a lower degree of intracellular specificity as compared to Poloxin. The faithful recapitulation of the phenotypic effect of the PBD overexpression by Poloxin in a strongly dose-dependent manner suggests that Poloxin interferes with correct localization of Plk1 by targeting the Plk1 PBD as the dominant mode of action.

In order to further validate that the mitotic arrest and the chromosome congression defects observed in the presence of the inhibitors were caused by incorrect distribution of endogenous Plk1, we analyzed the localization of Plk1 by immunofluorescence. In metaphase of DMSO-treated control cells, Plk1 located to centrosomes and kinetochores (Figure 3C). In contrast, in the presence of thymoquinone and Poloxin, both centrosomal and kinetochoral localization of Plk1 were significantly reduced in metaphase-arrested cells (Figure 3C). Similarly, centrosomal localization of Plk1 in prometaphase was strongly reduced in the presence of Poloxin and thymoquinone, but

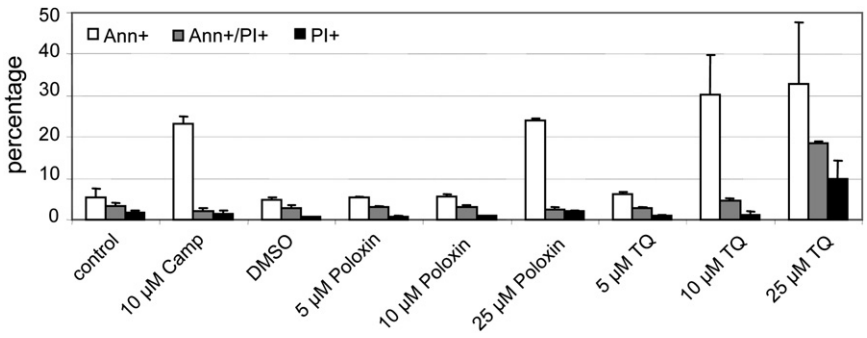

Figure 4. Poloxin and Thymoquinone Induce Apoptosis in HeLa Cells

HeLa cells were treated with Poloxin or thymoquinone (TQ) at the indicated concentrations for 24 hr. Nontreated cells (control), or cells treated with the apoptosis-inducing agent camptothecine (Camp), were analyzed for comparison. Annexin V and propidium iodide staining were performed as described in the Experimental Procedures. Annexin V staining alone (Ann+) indicates early apoptotic cells, while double staining by both Annexin V and propidium iodide (Ann+/PI+) indicates late apoptotic cells. Staining by propidium iodide alone (PI+) indicates necrotic cells. Error bars represent SD.

was not significantly affected in cells arrested in prometaphase by nocodazole treatment (Figure S3). Quantification of centrosomal Plk1 localization in prometaphase- and metaphase-arrested cells revealed a highly significant decrease in the presence of Poloxin and thymoquinone (Figure 3D). These data confirm the activities of the compounds against the function of the Plk1 PBD observed in vitro (Figures 1B and 1C; Table S1) in the cellular environment.

Inhibition of Plk1 functions by small-molecule inhibitors of its catalytic activity (Gumireddy et al., 2005; Lansing et al., 2007; Lenart et al., 2007; Liu et al., 2005; McInnes et al., 2006; Peters et al., 2006; Santamaria et al., 2007; Steegmaier et al., 2007; Stevenson et al., 2002) or RNAi-mediated depletion (Liu and Erikson, 2003; Spankuch-Schmitt et al., 2002a) induces apoptosis in cancer cells. In order to investigate whether interference with the intracellular localization of Plk1 is sufficient to cause apoptosis, unsynchronized HeLa cells were treated with Poloxin and thymoquinone, and were analyzed for Annexin V and propidium iodide staining by flow cytometry. The apoptotic frequency of HeLa cells was significantly increased in the presence of 25 μM Poloxin. Thymoquinone was found to increase the apoptotic frequency of HeLa cells already at 10 μM, but induced a significant degree of necrosis at 25 μM (Figure 4).

DISCUSSION

Plk1 is widely considered to be a target for anticancer therapy. A number of small-molecule inhibitors of the enzymatic function of Plk1 have been reported (Lansing et al., 2007; Lenart et al., 2007; Liu et al., 2005; McInnes et al., 2006; Peters et al., 2006; Santamaria et al., 2007; Steegmaier et al., 2007; Stevenson et al., 2002), which, with one exception (Gumireddy et al., 2005), are thought to exert their activities at least in part by competing for the ATP binding pocket. Clinical trials with small-molecule inhibitors of Plk1's catalytic activity are ongoing (Gumireddy et al., 2005; Steegmaier et al., 2007). Because the conserved nature of the ATP binding pocket poses a serious hurdle for the development of monospecific inhibitors, careful analysis of the compounds' activities on all kinases is necessary in order to select compounds which inhibit the right set of kinases. In fact, due to the redundancy of signaling pathways and the genetic instability of cancer cells, multitargeted inhibitors might be of superior therapeutic benefit than truly monospecific ligands of a kinase's ATP binding pocket (Daub et al., 2004). Nevertheless, the difficulties associated with the specificities of ATP-competitive kinase inhibitors encouraged us to explore an alternative mode of inhibition of Plk1.

The Plk1 PBD had been suggested as an ideal target for cancer therapy due to its unique nature which could facilitate the development of specific agents targeting its function (Elia et al., 2003b; Strebhardt and Ullrich, 2006). In this study, we explored the concept of inhibiting the Plk1 PBD by cell-permeable small molecules which prevent it from binding to its intracellular anchoring sites and substrates. Our data demonstrate that small molecules which interfere with the binding properties of the Plk1 PBD can cause mitotic arrest and apoptosis in cancer cells, and thus validate the Plk1 PBD as an anticancer target.

We chose the assay principle of fluorescence polarization (Owicki, 2000) for the primary screen, as this assay type is ideally suited to the identification of small-molecule inhibitors of binding between components of biological systems, such as inhibitors of DNA-protein interactions (Kiessling et al., 2006; Rishi et al., 2005) or peptide-protein interactions (Coleman et al., 2005; Schust et al., 2006). Recently, the scope of fluorescence polarization assays was expanded to screen for inhibitors of a protein-aptamer interaction, which led to the identification of a small-molecule inhibitor of a guanine nucleotide exchange factor (Hafner et al., 2006).

Despite thymoquinone's well-documented antineoplastic effects, clinical trials which could validate the benefit of thymoquinone or derivatives thereof for cancer patients have not been initiated. The lack of information about the molecular targets underlying thymoquinone's anticancer activity poses a serious hurdle to drug development—such knowledge would facilitate medicinal chemistry efforts aimed at improving the compound's pharmacological properties to the level required for drugs, and also the preselection of patients who are likely to respond in clinical trials (Corson and Crews, 2007). Thus, the discovery of the Plk1 PBD, and also of the STAT3 SH2 domain, as molecular targets of thymoquinone not only suggests a rational explanation for this natural product's antineoplastic effects (Gali-Muhtasib et al., 2006) but also provides a strong argument for the development of thymoquinone derivatives as anticancer drugs. Moreover, our data add thymoquinone to the very short list of natural products known to inhibit a protein-protein interaction. Nevertheless, it is to be expected that thymoquinone has additional molecular targets related to its anticancer activities that were not uncovered in this study. The PBD inhibitor Poloxin, a synthetic derivative of thymoquinone, already displays a superior specificity profile in vitro and in cells. In addition, Poloxin offers

the means of targeting the PBD on fast timescales, which should open the door for examining the function of the PBD in the context of cell-division dynamics. Finally, the specificity profiles of agents targeting the unique PBD like Poloxin are potentially more straightforward to analyze and manage than those of ATP-competitive kinase inhibitors. Thus, our study could have significant implications for anticancer therapy.

SIGNIFICANCE

Polo-like kinase 1 (Plk1) is a key player for multiple stages of mitosis, and has been established as a target for anticancer therapy. Usually, inhibitors of Plk1 target the conserved ATP binding site. In this manuscript, we provide proof of principle that Plk1 can alternatively be targeted by small molecules which inhibit the function of the polo-box domain (PBD), a recently discovered protein domain which mediates intracellular localization of Plk1. To our knowledge, the natural product thymoquinone and a synthetic derivative dubbed "Poloxin" identified by us are the first reported nonpeptidic inhibitors of the protein-protein interactions mediated by the Plk1 PBD. The compounds interfere with correct localization of Plk1, and thereby lead to mitotic arrest and apoptosis of cancer cells. Our data provide an explanation for the anticancer activity of the natural product thymoquinone, and make it one of the very few natural products known to inhibit a protein-protein interaction. Poloxin's more selective activity against the PBD should make it an excellent tool for analyzing the role of the PBD in mammalian cells, and has implications for the future design of anticancer drugs.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Protein Expression

DNA sequences coding for human Plk1 amino acids 326–603, human Plk2 amino acids 355–685, and human Plk3 amino acids 335–646 comprising the respective polo-box domains (Elia et al., 2003b) were amplified by PCR from plasmid DNA (Plk1) or placenta cDNA (Plk2, Plk3) and cloned into a modified pET28a (Plk1) vector, or into a modified pQE70 vector carrying a C-terminal 6× His tag and an N-terminal MBP tag (Plk2 and Plk3). Similarly, nucleotides coding for the forkhead-associated domain (FHA) of the kinase Chk2 (amino acids 1–225) (Durocher et al., 2000) and the WW domain of the proline isomerase Pin1 (amino acids 1–162) (Yaffe et al., 1997) were amplified by PCR from HeLa S3 and placenta cDNA, respectively, and cloned into a modified pET28a vector. Site-directed mutagenesis of the Plk1 PBD was applied to generate the inactive Plk1 PBD mutant displaying three mutations: W414F, H538A, and K540A (PBD FAA) (Schmidt et al., 2006). Wild-type and mutant PBD sequences were cloned into a modified pCS2 mammalian expression vector carrying a myc tag. Proteins were expressed from Rosetta BL21DE3 (Novagen) following the published procedure (Schust and Berg, 2004; Schust et al., 2006), purified by affinity chromatography, and dialyzed against a buffer containing 50 mM Tris (pH 8.0), 200 mM NaCl (for Plk1: 400 mM NaCl), 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.1% Nonidet P-40. The design of expression plasmids coding for STAT1, STAT3, and STAT5b as well as their expression and purification has been described (Schust and Berg, 2004; Schust et al., 2006).

High-Throughput Screening and Fluorescence Polarization Assays

Diverse chemical libraries consisting of 22,461 small molecules (8,298 molecules from ChemDiv, 9,000 molecules from Maybridge [Hits Kit 9000], and 5,163 miscellaneous organic molecules) were tested in a fluorescence polarization assay which analyzes binding between 5-carboxyfluorescein-GPMQSpTPLNG-OH (final concentration: 10 nM) and the Plk1 polo-box domain (final concentration: 65 nM). The final concentration of buffer components used was 10 mM Tris (pH 8.0), 50 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, and 10% DMSO. The following fluorophore-labeled peptides were used for specificity assays: Plk2: 5-carboxyfluorescein-GPMQTSpTPKNG-OH; Plk3: 5-carboxyfluorescein-GPLATSpTPKNG-OH; Chk2: 5-carboxyfluorescein-GHFDpTYLIRR-OH; Pin1: 5-carboxyfluorescein-GWFYpSPRLKK-OH; STAT1: 5-carboxyfluorescein-GpYDKPHVL-OH; STAT3: 5-carboxyfluorescein-GpYLPQTV-NH_2; STAT5b: 5-carboxyfluorescein-GpYLVLDKW-OH; Lck: 5-carboxyfluorescein-GpYEEIP-OH. Proteins were incubated at 22°C with the test compounds for 1 hr prior to addition of the fluorophore-labeled peptides, and fluorescence polarization was analyzed immediately thereafter. Proteins were used at the following final concentrations, which correspond approximately to the K_d values of the respective assays: Plk1: 65 nM; Plk2: 130 nM; Plk3: 1875 nM; Chk2: 240 nM; Pin1: 1000 nM; STAT1: 120 nM; STAT3: 160 nM; STAT5b: 110 nM; Lck: 40 nM. Fluorescein-labeled peptides were >95% pure. Inhibition curves were fitted using SigmaPlot (SPSS Science Software).

Chemical Compounds

Poloxin (**1**) was purchased from ChemDiv (compound code 1436-0018) and purified by reverse-phase HPLC. Identity and purity were furthermore verified by mass spectrometry and 400 MHz ^{1}H-NMR (>97%). Thymoquinone was purchased from Sigma-Aldrich, and its identity and purity were verified by mass spectrometry and 400 MHz ^{1}H-NMR (>97%).

Cell culture, transient transfection of cells, cellular extract preparation, and western blot analysis are described in the Supplemental Data.

Cell-Cycle Analysis

For the determination of mitotic indices shown in Figure 2A, the number of mitotic cells treated as described below under "Immunofluorescence Assay" within a population of 200–300 cells was counted. For the determination of the mitotic phase distribution shown in Figure 3A, populations of approximately 50 mitotic cells were analyzed. All experiments were performed in triplicate. For the cell-cycle analysis shown in Figures 2C and 2D, cells were harvested, washed with PBS, fixed in chilled 70% ethanol at 4°C for 30 min, treated with 1 mg/ml of RNase A (Sigma-Aldrich) for 30 min, and stained with 100 μg/ml of propidium iodide. The DNA content of 30,000 cells was determined with a FACScan flow cytometer (BD Biosciences). The data were analyzed with cell-cycle analysis software MODFIT LT 2.0 (Verity Software House). All experiments were performed in triplicate.

Apoptosis Assay

Apoptosis was assessed using Vybrant apoptosis assay kit 2 according to the manufacturer's instructions (Molecular Probes).

Immunofluorescence Assay

HeLa cells were grown on coverslips for 24 hr and arrested in G1/S by addition of 1 μg/ml aphidicolin for 14 hr. Cells were released into aphidicolin-free medium containing the indicated concentration of compounds at a final DMSO concentration of 0.5%. Nocodazole was used at a final concentration of 150 nM. After 7 hr, the medium was replaced with fresh medium which also contained the indicated concentration of compound. Fourteen hours after the initial release, cells were fixed and permeabilized in methanol for 15 min at −20°C. Cells were washed in PBS and incubated for 60 min in blocking solution (PBS with 0.1% Tween 20, 1% goat serum). All antibodies were diluted in blocking solution, and incubations were carried out for 1 hr at room temperature in a humidified chamber followed by three washes with PBS containing 0.1% Tween 20. The following antibodies were used: mouse monoclonal anti-γ-tubulin (1:1000; Sigma-Aldrich), which was detected with Alexa Fluor 546-conjugated goat anti-mouse (1:1000; Molecular Probes), mouse monoclonal anti-α-tubulin-FITC (1:500; Sigma-Aldrich), and rabbit polyclonal anti-Plk1 (1:75; Abcam), which was detected by Alexa Fluor 488-conjugated goat anti-rabbit (1:1000; Molecular Probes). DNA was stained with 4′,6-diamidino-2-phenylindole (2 μg/ml; Sigma-Aldrich). Immunofluorescence microscopy was performed on a DeltaVision microscope (Applied Precision) at 100× magnification. Images were processed using a deconvolution algorithm of Softworx software (Applied Precision). The centrosomal/cytoplasmic ratio of Plk1 staining was determined by measuring the total intensity in a circular

region of fixed diameter around the centrosome relative to the average intensity in three cytoplasmic regions of the same size. For quantification, nondeconvolved images with identical exposure times were used.

SUPPLEMENTAL DATA

Supplemental Data contain three figures, one table, and experimental procedures for cell culture, transient transfection of cells, cellular extract preparation, and western blot analysis and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/5/459/DC1/.

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