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Supporting Information

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Supporting Information

for

A Pan-Specific Inhibitor of the Polo-Box Domains of Polo-like Kinases Arrests Cancer Cells in Mitosis

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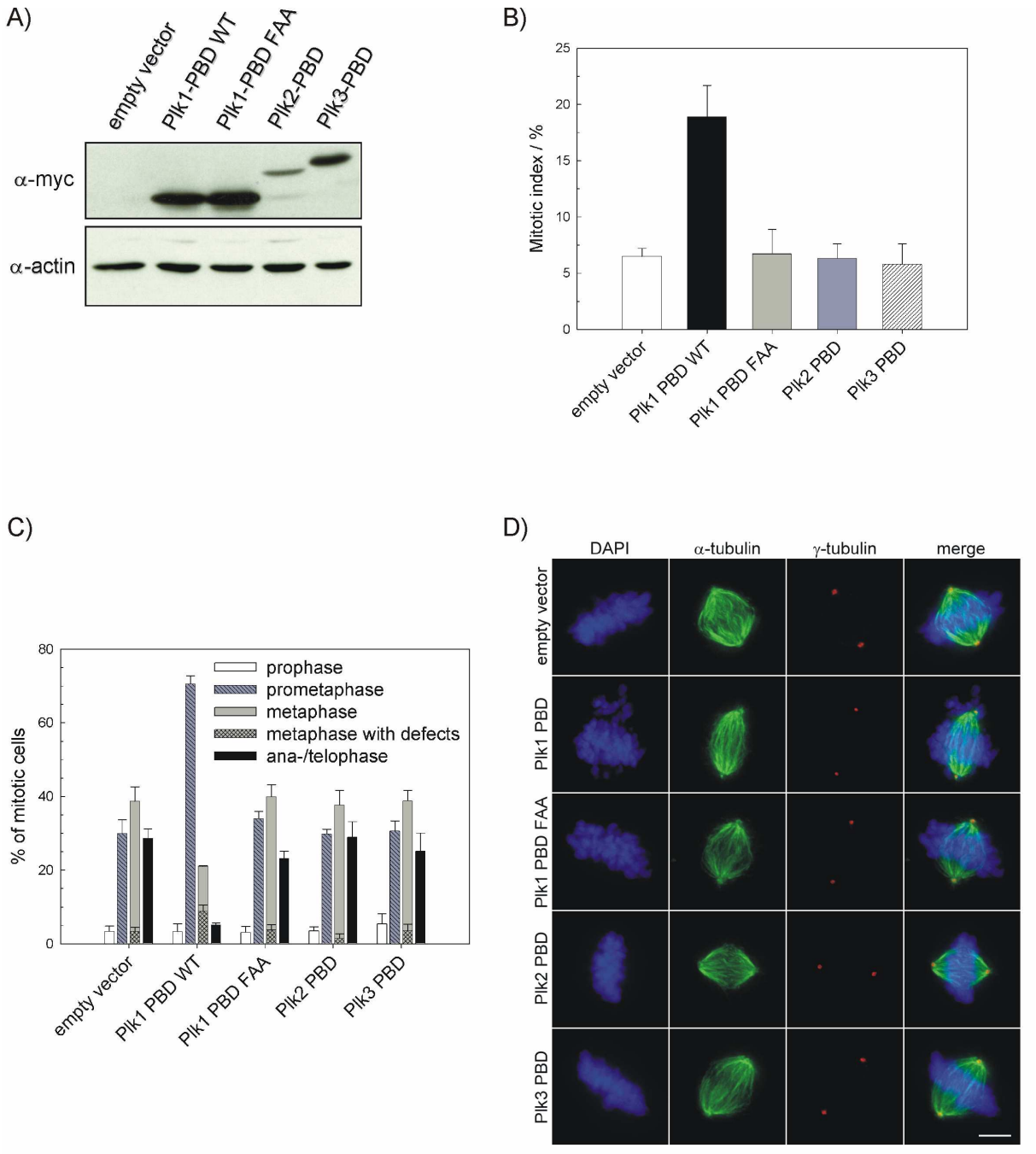

Figure S1. Overexpression of the Plk1 PBD, but not of the PBDs of Plk2 or Plk3, induces mitotic arrest in HeLa cells. A) Expression of Myc-tagged wild-type Plk1 PBD, an inactive triple mutant, wild-type Plk2 PBD, and Plk3 PBD in HeLa cells. B) Analysis of the percentage of cells arrested in mitosis (“mitotic index”). C) Analysis of the mitotic phases at which mitotic cells had been arrested by overexpression of the respective PBDs. D) Cellular phenotypes induced by overexpression of the respective PBDs. The scale bar represents 5 µm.

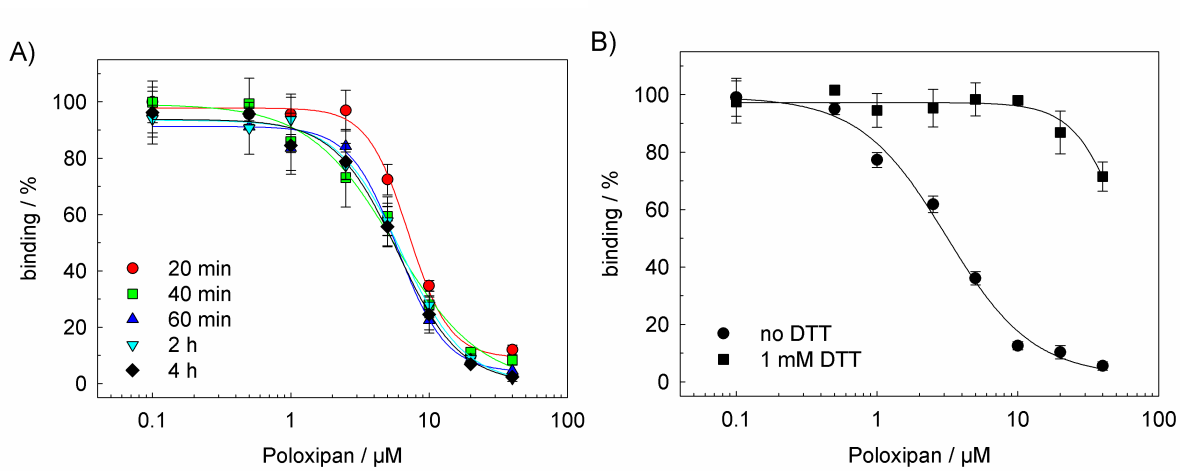

Figure S2. A) Inhibition of the Plk1 PBD by Poloxipan does not display significant time-dependence. The Plk1 PBD was incubated with Poloxipan at the indicated concentrations for the indicated periods of time at 22 ℃. Subsequentl y, the probe 5-carboxyfluorescein-GPMQSpTPLNG was added, and fluorescence polarization was analyzed immediately there-after. See Experimental procedures for details. B) Inhibition of the Plk1 PBD by Poloxipan is significantly decreased in the presence of 1 mM DTT in the assay buffer. See Experimental procedures for details.

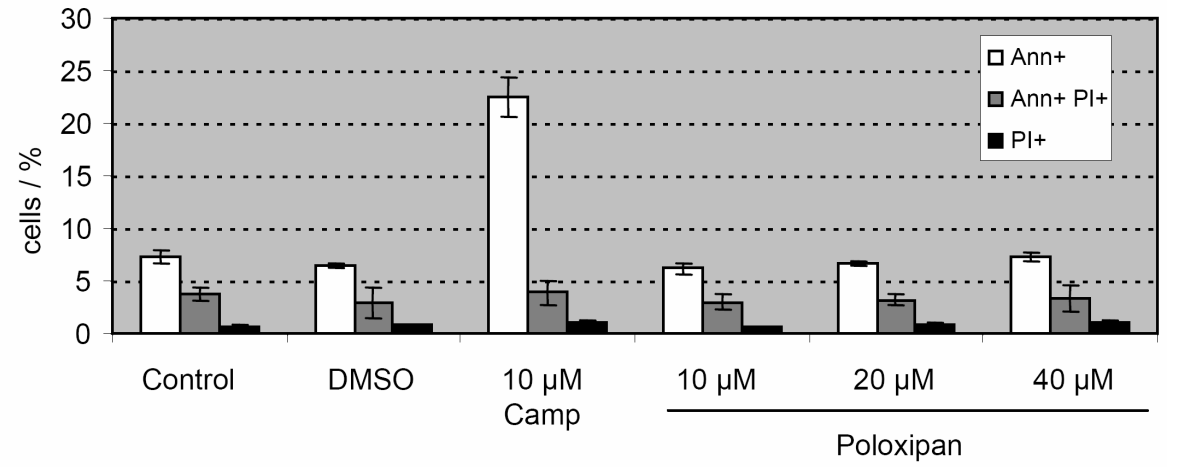

Figure S3. Poloxipan does not significantly increase the apoptotic rate of HeLa cells. HeLa cells were treated with Poloxipan at the indicated concentrations for 48 h. Nontreated cells (control), cells treated with the carrier DMSO only, or cells treated with the apoptosis-inducing agent camptothecine (Camp), were analyzed for comparison. Annexin V and propidium iodide staining were performed as described.[1] 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.

Experimental procedures

Plasmid construction and protein expression. Construction of the expression plasmids and protein expression have been described.[1] 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[2] 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 6xHis-tag and an N-terminal MBP-tag (Plk2 and Plk3). Similarly, nucleotides coding for the forkhead homology domain (FHA) of the kinase Chk2 (amino acids 1-225),[3] and for the proline isomerase Pin1 (amino acids 1-162)[4] 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 (PBD FAA)[5] displaying three mutations: W414F, H538A, and K540A. 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,[6,7] 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, STAT5b, and Lck as well as their expression and purification has also been described.[6,7] Design of expression plasmids coding for c-Myc, Max, and Jun and their expression has also been described.[8]

Fluorescence polarization assays. Experiments were essentially performed as described.**[1]** For the protein-peptide binding assays, the following fluorophore-labeled peptides were used (final concentration: 10 nM): Plk1: 5-carboxyfluorescein-GPMQSpTPLNG-OH; 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 ℃ with the test compoun ds for 1 h 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. 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. Fluorescein-labeled peptides were obtained from the core facility of the Max Planck Institute of Biochemistry, and by Peptide Specialty Laboratories (Heidelberg, Germany), and were > 95% pure. Protein-DNA binding assays for DNA binding of c-Myc/Max and Jun/Jun were essentially performed as described.[8] Inhibition curves were fitted using SigmaPlot (SPSS Science Software).

Chemical compound. Poloxipan was obtained from ChemDiv (compound code 5629-1160), and purified by chromatography (SiO_2 / ethyl acetate). Identity and purity were verified by 400 MHz ^{1}H-NMR and HPLC/HRMS (> 97%). The assignment of the *Z* configuration to the exocyclic double bond is based on information provided by the vendor and was not verified experimentally. However, 400 MHz ^{1}H-NMR showed only one major isomer, and the *Z* isomer is expected to be thermodynamically more stable.

Cell culture, transient transfection of cells, cellular extract preparation, and Western blot analysis were performed as described.[1]

Cell cycle analysis. For the determination of mitotic indices shown in Fig. 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 2B, populations of approximately 50 mitotic cells were analyzed. All experiments were performed in triplicate.

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

Immunofluorescence assay. HeLa cells were grown on coverslips for 24 h, and arrested in G1/S by addition of 1 µg/mL aphidicolin for 14 h. Cells were released into aphidicolin-free medium containing the indicated concentration of Poloxipan at a final DMSO concentration of 0.5%. After 7 h, the medium was replaced with fresh medium which also contained the indicated concentration of compound. 14 h 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 hour at room temperature in a humidified chamber, fol-

lowed by 3 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 (DAPI, 2 µg/mL, Sigma-Aldrich). Immunofluorescence microscopy was performed on a DeltaVision Microscope (Applied Precision) at 100x magnification. Images were processed using a deconvolution algorithm of the 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 round 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.

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