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

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Polo-like kinases (Plks) are a conserved family of serine/threonine kinases.[1,2] The family member Plk1 is a key regulator of mitosis[1,2] and has been identified as a negative prognostic marker for tumor patients.[3,4] The widespread recognition of Plk1 as a therapeutic target for the treatment of human tumors has triggered numerous drug discovery programs.[5–16] The vast majority of Plk1 inhibitors are thought to act on the ATP-binding pocket of the enzyme.[6–16] Due to the conserved nature of the ATP-binding pocket in the family of protein kinases and other ATP-binding enzymes, the development of monospecific, ATP-competitive inhibitors is an enormous challenge.

In addition to their catalytic domains, polo-like kinases harbor conserved regions termed polo boxes in their C terminus.[17] Plk1, Plk2, and Plk3 each have two polo boxes, which, in combination with an N-terminal linker domain, form the polo-box domains (PBDs). PBDs bind to short, contiguous peptide sequences that bear a phosphorylated serine or threonine residue.[17,18] Plk4 is unique in that it only carries a single polo box that forms intermolecular dimers to constitute a functional PBD,[19] but it is currently unclear whether this domain also binds to phosphorylated peptide motifs. Since the PBD of Plk1 was shown to be essential for mitotic progression, inhibition of the PBD of Plk1 had been suggested as an alternative approach to the inhibition of Plk1 by small molecules.[3,18] It is conceivable that inhibitors of the Plk1 PBD will be less prone to selectivity issues than ATP-competitive inhibitors,[20] because there are only three (or four) PBDs in mammals. Following this logic, we have recently reported that the natural product derivative poloxin is a small-molecule inhibitor of the Plk1 PBD.[21] Subsequently, Watanabe and co-workers reported the natural product purpurogallin as an inhibitor of the Plk1 PBD.[22] Purpurogallin and poloxin exhibit similar in vitro specificity profiles in that they preferentially target the PBD of Plk1, affect the PBD of Plk2 to a lesser extent, and exert only a minor effect on the PBD of Plk3. None of the compounds was tested against the PBD of Plk4 dimers because of Plk4's unclear binding preferences. The similarity of the optimal binding peptides of the PBDs of Plk1-3, which share a SpTP core element,[18] suggests that absolute specificity for the Plk1 PBD over the PBDs of Plk2 and Plk3 may be difficult to achieve.

The requirement of selectivity for Plk1 PBD inhibitors to be used as anticancer agents with respect to the PBDs of Plk2 and Plk3 is currently unclear. While it has been shown that the PBDs of Plk2[23,24] and Plk3[25] mediate intracellular localization of the enzymes, the cellular consequences of inhibiting their functions with small molecules have not been fully explored. The PBD of Plk2 has been shown to be essential for centrosomal localization of Plk2 and centriole duplication.[24] However, because Plk2$^{-/-}$ embryos are viable,[26] and Plk2 depletion in HeLa and U2OS cells does not alter progression through the cell cycle,[27] it can be speculated that inhibition of the Plk2 PBD by an anticancer drug may not have drastic side effects. The effects of Plk3 mislocalization by over-expression of its PBD are discussed controversially in the literature; in one study over-expression of the Plk3 PBD induced mitotic arrest,[25] but not in another.[28] It is likely that these partially divergent results are due to different expression levels of the various PBD constructs used between the studies. In our hands, over-expression of the PBDs of Plk3 (and Plk2) did not have a significant effect on the mitotic indices and the mitotic subphases of HeLa cells (Figure S1 A–C in the Supporting Information). In contrast, mislocalization of Plk1 induced by over-expression of its PBD strongly induced mitotic arrest of HeLa cells, mostly in prometaphase, and led to characteristic chromosome congression defects, consistent with the literature (Figure S1).[21,22,25,28,29] These data suggest that concomitant inhibition of the PBDs of Plk2 and Plk3 by an inhibitory agent targeted against the Plk1 PBD would not strongly interfere with the inhibitor's ability to induce mitotic arrest in cancer cells.

In this study, we aimed to investigate the cellular effects of a pan-specific inhibitor of the PBDs of Plk1, Plk2, and Plk3. Screening of chemical libraries against the PBD of Plk1 has provided us with several hit compounds.[21,30] Profiling of their activities against the PBDs of Plk2 and Plk3 and selection of compounds with activities against all three PBDs led us to identify poloxipan (for "polo-box domain inhibitor, pan-specific"; Figure 1 A, **1**). Poloxipan inhibited the function of all three PBDs with IC_{50}s in the single-digit micromolar concentration range in fluorescence polarization assays, and inhibited the PBD of Plk2 even slightly better than the PBD of Plk1 (IC_{50} values: Plk1 PBD: 3.2 ± 0.3 μM; Plk2 PBD: 1.7 ± 0.2 μM; Plk3 PBD: 3.0 ± 0.1 μM; Figure 1 B). Binding of other peptide motifs bearing phosphorylated threonine and serine residues to the FHA domain of the checkpoint kinase Chk2, or to the peptidylprolyl *cis/trans* isomerase Pin1, was inhibited only to a significantly

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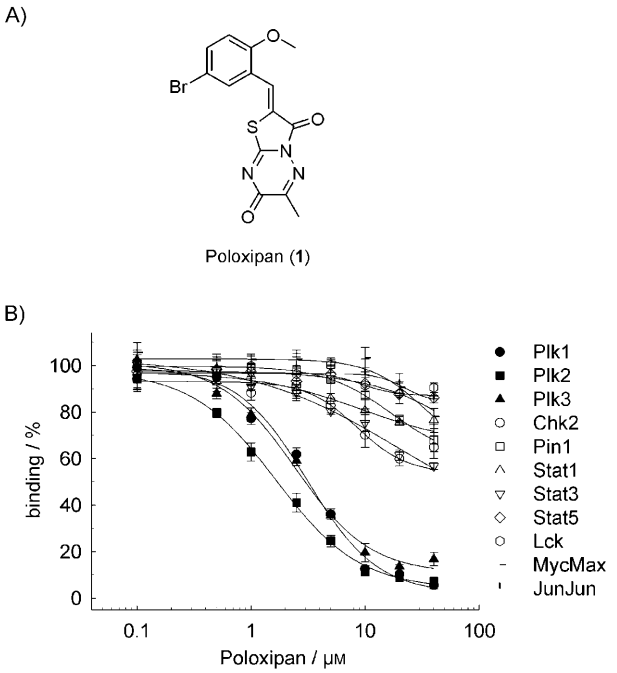

Figure 1. A) Structure of poloxipan. B) Poloxipan inhibits the functions of the PBDs of Plk1-3 in fluorescence polarization assays.

lesser extent (maximum inhibition at 40 μM: Chk2 FHA: 35 ± 5%; Pin1: 32 ± 5%), indicating that poloxipan is not a general inhibitor of phosphoserine/phosphothreonine binding domains. Similarly, the functions of the phosphotyrosine-binding Src homology (SH) 2 domains of STAT1, STAT3, STAT5b, and Lck were inhibited only to a minor extent (inhibition at 40 μM: 24 ± 5%, 43 ± 2%, 14 ± 1%, and 10 ± 2%, respectively). Moreover, dimerization and/or DNA binding of the structurally unrelated dimeric transcription factors c-Myc/Max and Jun/Jun, which served as controls for nonspecific effects, was inhibited only to a minor extent (maximum inhibition at 40 μM: c-Myc/Max: 22 ± 4%; Jun/Jun: 14 ± 2%). The use of higher concentrations of poloxipan was prevented by its limited solubility in aqueous buffers. Inhibition of the Plk1 PBD by poloxipan did not significantly increase over time; this argues against the formation of a covalent bond between the proteins and the inhibitor despite the presence of a Michael acceptor system in its core structure (Figure S2 A). However, the compound's activity was sharply decreased in the presence of dithiothreitol (DTT) in the assay buffer, indicating that the inhibitor is amenable to attack by nucleophilic agents (Figure S2 B).

Inhibition of Plk1 in cancer cells by antisense oligonucleotides,[31] small interfering RNAs,[32,33] a Plk1-derived peptide,[34] small-molecule inhibitors of its catalytic activity,[5–13] or selective inhibitors of its PBD[21,22] has been uniformly demonstrated to arrest cells in mitosis. In order to test whether a pan-specific PBD inhibitor can also arrest tumor cells in mitosis, cells derived from a cervical cancer patient (HeLa), which had been synchronized at the G1/S transition, were released from cell cycle block into tissue culture media containing poloxipan. Analysis of mitotic cells was performed 14 h after release, when most control cells had successfully completed mitosis already (less than 5% of control cells are in mitosis after 14 h). We observed a dose-dependent increase in cells arrested in mitosis (Figure 2 A). The effects of poloxipan were much more pronounced when the tissue culture media containing the inhibitor was exchanged for fresh media (also containing the inhibitor) 7 h after release from G1/S arrest (data not shown), presumably because the compound is unstable under the tissue culture conditions, but further studies would be required to verify this hypothesis. Dissection of the mitotic phases at which individual cells had been arrested indicated a predominant prometaphase arrest, as was observed with the more selective Plk1 PBD inhibitors[21,22] (Figure 2 B). Furthermore, poloxipan increased the incidence of chromosome congression defects (one or several chromosomes of cells arrested in metaphase fail to congress to the metaphase plate) similar to poloxin[21] and purpurogallin.[22] Within the population of cells in metaphase, the percentage of cells with chromosome congression defects increased from 9% in DMSO treated cells to 14%, 25%, and 53% in cells treated with 10 μM, 20 μM, and 40 μM of poloxipan, respectively (Figure 2 B, C). Within the population of prometaphase and metaphase cells with chromosome congression defects, we observed a highly significant decrease in centrosomal localization of Plk1 as compared to control cells treated with DMSO only ($p < 0.001$); this supports the notion that the function of the Plk1 PBD is inhibited by poloxipan (Figure 2 D, E).

Of note, 33 ± 8% of metaphase cells with chromosome congression defects in the presence of 40 μM poloxipan displayed fragmented centrosomes (Figure 3). Fragmented centrosomes were not observed in poloxipan-treated metaphase cells without chromosome congression defects. These findings appear to be related to the findings of Watanabe and co-workers, who reported that centrosomes at the spindle poles in cells treated with the Plk1 PBD inhibitor purpurogallin were frequently unfocused and more distanced than in control cells.[22] The underlying molecular cause for centrosome fragmentation in cells treated with poloxipan will need to be clarified in future studies. Because depletion or inhibition of phosphorylation of the centrosomal Plk1 substrate Kizuna causes fragmentation and dissociation of the pericentriolar material from centrioles, and Kizuna appears to bind to the PBD of Plk1,[35] inhibition of the Kizuna/Plk1 interaction by poloxipan could be a potential explanation for centrosome fragmentation. However, the inhibition of additional targets as the underlying molecular reason for centrosome fragmentation cannot be fully excluded based on the present data. Poloxipan did not significantly increase the percentage of apoptotic cells as compared to DMSO-treated control cells, presumably due to lack of stability over the time course of the assay of 48 h (Figure S3).

In summary, we have identified poloxipan as a small molecule that inhibits the function of the PBDs of Plk1–3 to similar extents in vitro, and have demonstrated that its cellular effects are very similar to the reported effects of compounds that target the Plk1 PBD more selectively.[21,22] Although it is tempting to speculate that inhibitors of the Plk1 PBD to be used as antineoplastic agents in the clinic may not necessarily need to be optimized for minimal activity against the PBDs of Plk2 and

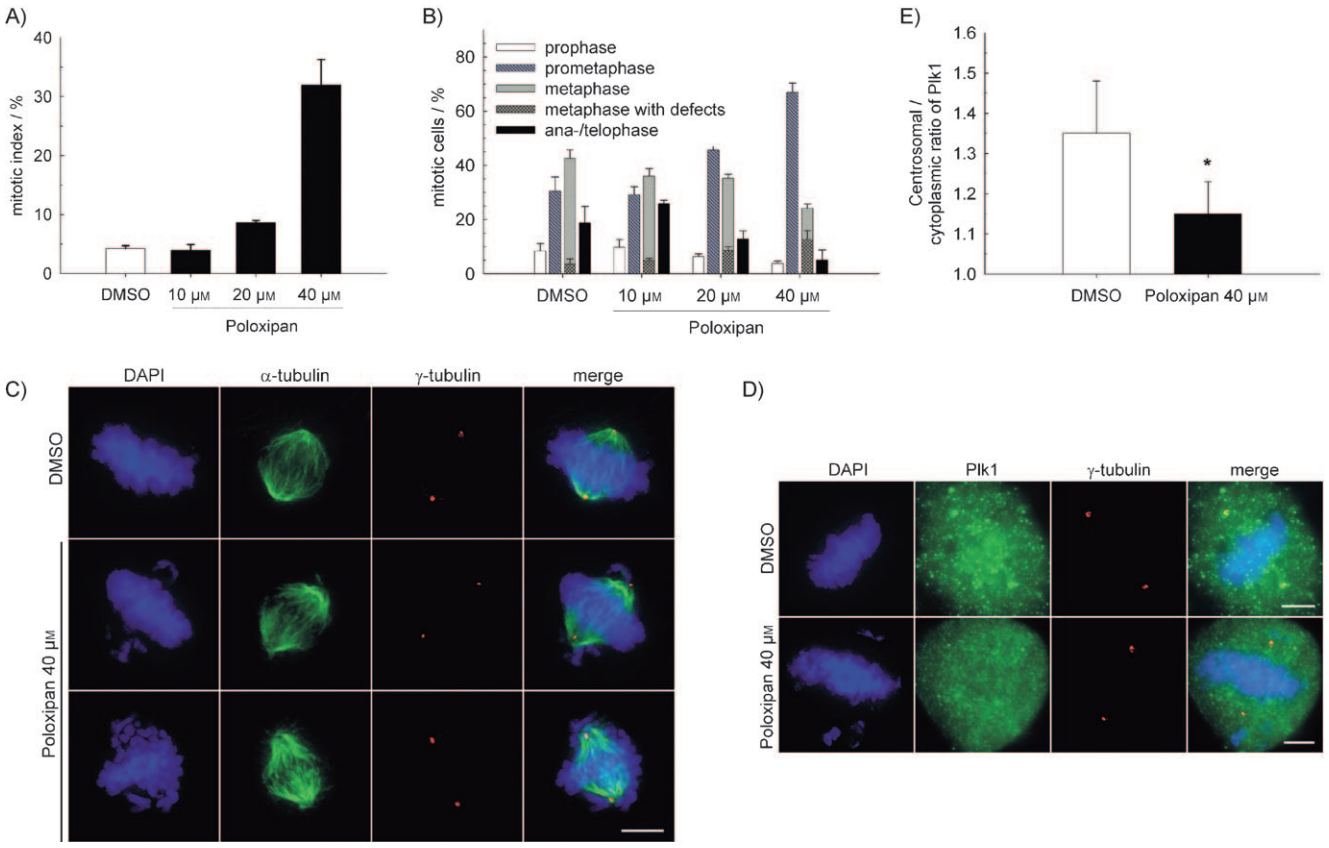

Figure 2. A) Poloxipan induces mitotic arrest in HeLa cells. B) Distribution of mitotic phases and chromosome congression defects within the total metaphase population in the presence of poloxipan. Populations of approximately 50 mitotic cells were analyzed ($n = 3$). C) Poloxipan induces chromosome congression defects in HeLa cells. The scale bar represents 5 μm. D) Poloxipan interferes with localization of Plk1 to the centrosomes in metaphase cells with chromosome congression defects. The localization of the centrosomes is indicated by the γ-tubulin staining. In DMSO-treated cells, the γ-tubulin staining coincides with distinct signals generated by the anti-Plk1 antibody; this leads to yellow signals in the merged pictures. In cells treated with poloxipan, the γ-tubulin staining coincides with the anti-Plk1 staining only to a significantly lower extent. The scale bars represent 5 μm. E) Quantitative analysis of the effects of poloxipan on centrosomal Plk1 localization in prometaphase or metaphase cells with chromosome congression defects. For each treatment regime, 20 centrosomes from ten cells were analyzed. *$p < 0.001$, calculated using Student's t test (unpaired, two-sided).

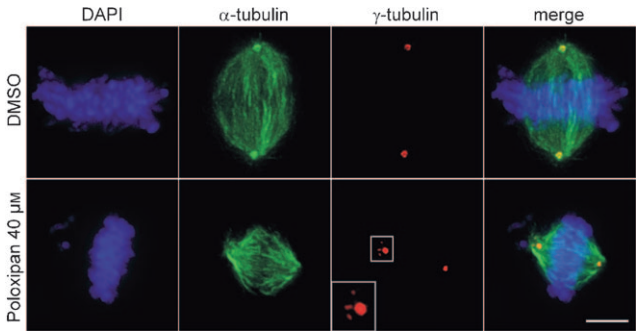

Figure 3. Fragmentation of centrosomes in metaphase cells with chromosome congression defects in the presence of poloxipan. The localization of the centrosomes is indicated by the γ-tubulin staining. The scale bar is 5 μm. The content of the frame in the γ-tubulin stain is magnified by a factor of two.

Plk3, further investigation of this question in animal studies is warranted. Of note, Plk3$^{-/-}$ mice have been reported to display increased weight and to develop tumors more frequently than wild-type littermates.[36] Animal studies with selective inhibitors of the Plk3 PBD could clarify whether inhibition of the Plk3 PBD, without inhibiting Plk3's enzymatic activity, is tolerated more favorably than the genetic knockout. Our data provide increasing evidence that the inhibition of the Plk1 PBD by small molecules is feasible, and that it provides an attractive alternative to the inhibition of Plk1 by ATP-competitive inhibitors.

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