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Enabling and disabling Polo-like kinase 1 inhibition through chemical genetics

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Abstract

Polo-like kinase 1 (Plk1) is a core regulator of cell division and an emerging target for cancer therapy. Pharmacologic inhibitors of Plk1 exist but affect other kinases complicating their in vivo validation. To address this, we examined effects of two structurally unrelated Plk1 inhibitors (BI-2536 and TAL) against isogenic human cell lines that solely express wildtype (wt) or analog-sensitive (as) Plk1 alleles. Unexpectedly, Plk1^{as} cells displayed profound biochemical and functional resistance to both inhibitors. Cells that co-express Plk1^{wt} and Plk1^{as} exhibit loss-of-function phenotypes only when both kinase alleles are inhibited. Resistance to BI-2536 is linked to an intragenic suppressor mutation (C67V) that restores an otherwise invariant valine to the kinase active site. Structural modeling demonstrates that this mutation not only enables Plk1^{as} to function in vivo, but also occludes BI-2536 from the ATP-binding pocket. Our results reveal the molecular basis of Plk inhibitor selectivity and a potential mechanism for tumor cell resistance.

Keywords

orthogonal control; mitosis; chemical biology; Plk1

Polo-like kinase 1 (Plk1) is an important target for anti-neoplastic therapy because of its required functions in cell division,(1, 2) as well as the enhanced sensitivity of cancer cells to its inactivation.(3–5) Several Plk1-targeted drugs have emerged, including BI-2536(6) and ZK-thiazolidinone (TAL).(7) These inhibitors of Plk1 arrest cells in mitosis and evoke phenotypes consistent with downregulating Plk1 by other means.(1, 2) BI-2536 and a pharmacologically optimized analog (BI-6727) have shown promising signs of activity in preliminary clinical trials.(8, 9) A chemical genetic system for inhibiting Plk1 has also been developed.(10) In this system, both copies of the *PLK1* locus were deleted from immortalized human retinal pigment epithelial cells through targeting and Cre-lox mediated recombination. After Cre-mediated excision, *PLK1*^{-/-} clones are inviable unless they are complemented by expression of Plk1 in *trans*, either wildtype (Plk1^{wt}) or a compound mutant (L130G C67V; hereafter Plk1^{as}). By enlarging the kinase's active site, the latter mutations allow Plk1 to accept bulky purine analogs as ATP-competitive inhibitors. We

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report here that these mutations also have the unexpected effect of desensitizing Plk1 to clinically useful inhibitors like BI-2536 and TAL. (Structures for all chemicals used are shown in Supplementary Figure 1.)

In the course of examining the anti-proliferative activity of BI-2536, we discovered that this compound strongly retarded the growth of Plk1^{wt} cells, but had little or no effect on Plk1^{as} cells (Figure 1A–B). To determine if this change in inhibitor potency was unique to BI-2536, we performed similar growth-challenge experiments with TAL, a structurally distinct Plk1 inhibitor. Again, we observed a marked difference between Plk1^{as} cells and their isogenic Plk1^{wt} counterparts (Figure 1C–D). To understand the depth and breadth of inhibitor resistance, we queried multiple *in vivo* readouts of Plk1 activity. Plk1 is required throughout mitosis, with well-characterized roles in centrosome maturation, bipolar spindle assembly, stabilization of kinetochore-microtubule attachments, and initiation of cytokinesis. Each of these programs proved to be qualitatively and quantitatively resistant to both Plk1-targeted inhibitors. For instance, Plk1^{as} cells continued to recruit γ -tubulin to centrosomes (a cardinal manifestation of centrosome maturation) and form bipolar spindles in the presence of BI-2536 (Figure 2A) and TAL (Figure 2B). Likewise, BubR1 hyperphosphorylation by Plk1 (a crucial determinant of stable kinetochore-microtubule attachment) was undiminished, as reflected in the BubR1 polypeptide's persistent mobility shift on SDS-PAGE (Figure 2C). Consistent with this broad array of defects, both compounds caused Plk1^{wt} (but not Plk1^{as} cells) to arrest in mitosis, as judged from their rounded appearance by phase-contrast microscopy (shown below in Figure 4).

Unlike conventional genetic probes, small-molecule inhibitors provide fine temporal control over Plk1 inhibition, a property that has been leveraged to expose the kinase's previously unexplored roles in late mitosis (i.e., downstream of the spindle assembly checkpoint), simply by deferring inhibitor treatment until the metaphase-to-anaphase transition.^(10, 11) Using this timed approach, we discovered that BI-2536 is unable to block Plk1's relocalization to the spindle midzone and induction of cytokinetic furrows in Plk1^{as} cells (Figure 2D). Crucially, in this and all other assays, we verified that Plk1^{as} cells were nonetheless sensitive to the bulky purine analog 3-MBPP1, demonstrating that they had not simply bypassed the need for Plk1 altogether (for instance, through overexpression or mutation of another Plk family member).

In classical genetics, allelism tests are used to determine if two alterations (for instance, two temperature-sensitive mutations) target the same gene or different genes. To extend this principle to chemical biology, we re-introduced Plk1^{wt} into Plk1^{as} cells and then repeated the battery of tests described above. In all cases Plk1^{wt/as} cells proved to be resistant to 3-MBPP1 and BI-2536 when applied individually but not simultaneously (Figure 3). This result validates 3-MBPP1 and BI-2536 as the chemical equivalents of alleles—that is, their effects on mitosis and cell division arise through their common target Plk1, rather than any non-overlapping targets of either compound. Moreover, this reveals that catalytically inactive Plk1 alleles do not confer dominant negative phenotypes when expressed at near-physiologic concentrations. Taken together, these findings empower the orthogonal control of Plk1 activity and highlight a general principle of chemical genetics that can be used to compare and contrast any pair of inhibitors. This is analogous to the powerful set of mTOR mutants and cognate rapalogs which can be controlled in an orthogonal manner.⁽¹²⁾

We noted that BI-2536 eventually inhibited the growth of Plk1^{as} cells at high concentrations (Figure 1B). To determine whether this growth inhibition correlated with eventual inactivation of Plk1^{as} per se, we performed cell cycle analysis (Supplementary Figure 2). Whereas BI-2536 caused a mitotic arrest in Plk1^{wt} cells at nanomolar concentrations, no similar arrest was ever seen Plk1^{as} cells, even at micromolar concentrations. Rather, these

saturating conditions provoked a G1/S delay that is inconsistent with Plk1's known functions and moreover independent of Plk1's mutational status. We conclude that Plk1 ceases to be BI-2536's dose limiting target *in vivo* once endowed with these active site alterations.

We next sought to understand the structural basis of Plk1^{as} resistance. A crystallographic model of BI-2536 bound to Plk1 previously revealed the importance of multiple residues responsible for the selectivity of this compound, including both Cys67 and Leu130.(13) In order to determine whether resistance of Plk1^{as} cells to this compound is attributable to L130G, to C67V, or to both mutations, we examined the crystallographic model of Plk1 bound to BI-2536. As noted by Kothe et al.,(13) Cys67 and Leu130 contact the cyclopentyl and ethyl moieties of this inhibitor (Figure 4A). Modeling of mutations reveals that the Leu130 'gatekeeper' mutation can potentially weaken a van der Waals interaction whereas C67V mutation results in steric clash with the cyclopentyl group (Figure 4B). To determine which is the primary driver of resistance, we tested the properties of the single mutants. Plk1^{L130G} was unable to rescue *PLK1*^{-/-} cells, demonstrating that this 'gatekeeper' mutation disables Plk1 if separated from its intragenic suppressor, which is thought to stabilize an antiparallel β -sheet in the kinase's N-terminal lobe. In contrast, Plk1^{C67V} was not only functional but also highly resistant to BI-2536, as judged from cell-growth experiments (Figure 4C) and *in vitro* kinase assays (Figure 4D). Interestingly, nearly all other protein kinases contain valine at this position,(14) so Plk1 inhibitors can achieve selectivity by molecular recognition of cysteine at this site.(13)

We next sought to determine if the C67V mutation is sufficient to establish resistance to clinically relevant inhibitors of Plk1 other than BI-2536. We challenged cells harboring different Plk1 alleles with the structurally-related compound BI-6727, as well as with structurally distinct TAL and GSK461364, and assessed whether there was an increased number of round-appearing mitotic cells by phase-contrast microscopy (Figure 4E). This assay demonstrated that cells expressing Plk1^{C67V} were indeed resistant to BI-6727 and GSK461364, and partially resistant to TAL. Cell proliferation assays were consistent with these findings for BI-2536 and BI-6727 (Supplementary Figure 3). Thus several distinct Plk1 inhibitors in clinical development appear to exploit the C67 residue for selectivity and mutation can confer broad resistance.

Establishing the selectivity of protein kinase inhibitors is often challenging, as the active sites of the ~500 members of the human kinome possess a high degree of homology. While *in vitro* binding and activity assays provide some useful information in this regard, such readouts may or may not correlate with inhibitor selectivity *in vivo*, due to differences in enzyme or substrate abundance, post-translational modification, subcellular localization, or counteraction by other enzymatic pathways (e.g., protein phosphatases). Here, we have used chemical genetics to identify mutations that disable Plk1's inhibition by two structurally unrelated compounds, while enabling its orthogonal inhibition by a third class of small molecules. This is useful to validating BI-2536 and TAL as basic research tools. However, such active-site plasticity could limit the usefulness of these compounds in cancer therapy if tumor cells are able to escape these inhibitors by mutating Plk1's atypical but non-essential cysteine to a bulkier residue. Genetic surveillance of tumors that relapse during Plk1-targeted therapy is therefore warranted.

METHODS

Cell culture, transgenesis, and imaging

Cell lines were derived from hTERT-RPE1 (ATCC, Manassas, VA) propagated in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 2.5 mM L-glutamine, 10%

fetal bovine serum and 100 units/ml penicillin-streptomycin. Plk1^{as} cells and of GFP-Plk1^{as}/mCherry Plk1^{wt} were previously developed, and cells expressing Plk1^{C67V} were derived for this study in a similar manner.(10, 11) For proliferation assays, 2500 cells were plated into 24-well cell culture plates, cultured for 7 days, fixed and stained for viability with crystal violet as previously described.(10, 11)

For immunofluorescence cells were seeded into chamber slides (Nunc) and fixed after the appropriate treatments in methanol. Indirect immunofluorescence was performed as described previously.(10) For analysis of anaphase phenotypes, cells were arrested in mitosis with monastrol for 8 hours, washed, and released into fresh medium, followed by addition of 3-MBPP1 or BI-2536 30 minutes later. After twenty minutes of drug treatment, cells were fixed and processed for immunofluorescence. Fixed cells were visualized on a Nikon TE2000 inverted fluorescence microscope.

Immunoprecipitation and blotting

For immunoprecipitation kinase assays, cells expressing transgenic Plk1^{wt} and Plk1^{C67V} (Figure 4D) were arrested in mitosis with nocodazole and collected. Immunoprecipitation-kinase assays were performed as described previously, using anti-Plk1 antibodies (Zymed). (10) Equal loading was verified by coomassie. To analyze BubR1 phosphorylation, cells in log-growth phase were treated with nocodazole alone or in combination with 200 nM BI-2536, 10 μ M 3-MBPP1, or 1 μ M TAL for 15h, then collected by trypsinization. Cells were pelleted, frozen, and resuspended in buffer as above. Equivalent amounts of extract were loaded on to a 10% SDS-PAGE gel and separated by electrophoresis, followed by transfer to PVDF and immunoblotting as previously described. (10)

Cell Cycle analysis

To evaluate cell-cycle effects of BI-2536, cells were plated in 75 cm² in flasks and grown to log-growth phase (~60% confluence), followed by addition of fresh medium containing the appropriate concentration of BI-2536 or DMSO. Cells were collected and divided into two parts for propidium iodide-FACS analysis of DNA content and for staining with Hoechst 33258 to determine mitotic index by microscopic analysis of chromosome condensation. The fraction of cells in G2 was calculated as the fraction of 4N cells not in mitosis.

Antibodies and chemicals

Chemicals used in this study include monastrol (Calbiochem, standard working concentration 100 μ M), nocodazole (Sigma, 0.2 mg/ml), BI-2536 (200 nM or as indicated), 3-MBPP1 (10 μ M), ZK-TAL (1 μ M), BI-6727 (Selleck, 200 nM), GSK461364 (Chemietek, 2 μ M). Antibodies used in this study: BubR1 (Chemicon) 1:1000 WB; dsRed (Detects mCherry; Clontech) 1:1000 IF; GFP clone 3E6 (Invitrogen) 1:1000 IF; Plk1 cocktail (Zymed) for immunoprecipitation; α -tubulin (Chemicon) 1:1000 IF; γ -tubulin (Sigma) 1:200 IF.

Molecular modeling

A crystal structure of BI-2536 bound to Plk1 was obtained from the protein databank, accession 2RKU. (13) Residue mutations were made using the SwissProt PdbViewer (15) without energy minimization and surfaces were displayed with Accelrys DS Visualizer (Accelrys Software, San Diego).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Plk1^{as} cells can proliferate in the presence of BI-2536 and TAL. a–b) Comparison of cell line proliferation in the presence of 3-MBPP1 (10 μ M), BI-2536 (200nM or as shown). c–d). Proliferation assay in presence of 3-MBPP1 or TAL.

Figure 2. BI-2536 and TAL fail to induce Plk1 loss of function phenotypes in *Plk1^{as}* cells. a–b) Mitotic spindles after 3h incubation with the chemical noted. Percentage of spindles with monopolar phenotype is shown for conditions where this phenotype exceeded 2%. c) BubR1 hyperphosphorylation in *Plk1^{wt}* and *Plk1^{as}* cells in presence of 3-MBPP1 (3-MB), BI-2536 (BI) and TAL. d) Anaphase and cytokinesis phenotypes determined by Plk1 immunofluorescence in anaphase cells. When Plk1 is inhibited, cells lack furrows and fail to recruit Plk1 to the spindle midzone (arrowheads). Scale Bars, 10 μ M.

Figure 3.

Wildtype and analog-sensitive alleles of Plk1 are orthogonal in cells that express both $Plk1^{as}$ and $Plk1^{wt}$. a) Model and predicted drug sensitivity for human cells that contain both Plk1 alleles. b) Proliferation assay demonstrating the sensitivity of human RPE cells expressing both $Plk1^{wt}$ and $Plk1^{as}$ to 3-MBPP1 and BI-2536 separately and together. c) Phase contrast image of asynchronously growing cells expressing both $Plk1^{as}$ and $Plk1^{wt}$ after drug challenge for 14 hours. An increase of round-appearing mitotic cells is seen only in the presence of both 3-MBPP1 and BI-2536. d–e). Spindle structure and BubR1 phosphorylation assays performed as described in Figure 2, stained with DAPI (blue), γ -tubulin (green) and α -tubulin (red). f) Cytokinesis assay as in Figure 2D. Individual Plk1 alleles are distinguished through tags: GFP ($Plk1^{as}$) and mCherry ($Plk1^{wt}$). In the presence of 3-MBPP1 or BI-2536, both alleles are recruited to the midspindle. When both are inhibited, neither are recruited and furrow formation fails (arrowheads). Scale Bars, 10 μ M.

Figure 4.

The C67V mutation of Plk1 is sufficient to impart resistance to BI-2536. a) Crystal structure of BI-2536 bound to wild type Plk1. Cysteine 67 (blue) interdigitates between the ethyl and cyclopentane moieties of BI-2536, whereas Leu130 (green) contacts the ethyl group. b) The C67V mutation (red) results in steric clash (yellow dashed lines) with both the ethyl and cyclopentyl groups by virtue of the greater breadth of valine than cysteine. L130G reduces contact with BI-2536 but does not clash (green). c) Cells with Plk1^{C67V} are resistant to BI-2536 in proliferation assays at nearly the same concentrations seen in Plk1^{as} cells. d) Immunoprecipitation-kinase assay demonstrates that Plk1^{C67V} is sufficient to provide resistance to BI-2536; 50% inhibitory concentrations (IC₅₀) are shown. e) Survey of sensitivity of cell lines to multiple inhibitors of Plk1 in clinical development. Phase contrast image of asynchronously growing cells expressing Plk1^{as}, Plk1^{wt}, or Plk1^{C67V} after challenging with the chemical indicated for 8 hours. Mitotic round cells increase when Plk1 is inhibited.