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Selectivity-determining Residues in Plk1

Michael Kothe, Darcy Kohls, Simon Low, Rocco Coli, Glen R. Rennie, Frederic Feru, Cyrille Kuhn and Yuan-Hua Ding*

Pfizer Global Research and Development, Research Technology Center, 620 Memorial Drive, Cambridge, MA 02139, USA
**Corresponding author: Yuan-Hua Ding, yuan-hua.ding@pfizer.com*

Polo-like kinase 1 is an important regulator of cell cycle progression whose over-expression is often associated with oncogenesis. Polo-like kinase 1 hence represents an attractive target for cancer intervention. BI 2536 (Boehringer Ingelheim, Ingelheim, Germany), a Polo-like kinase 1 inhibitor currently in clinical trials, exhibits nanomolar potency against Polo-like kinase isoforms and high selectivity against other kinases. We have previously published the crystal structures of the Polo-like kinase 1 domain in complex with AMPPNP and an Aurora A inhibitor. In this work, we present the co-crystal structure of Polo-like kinase 1 with BI 2536. The structure, in combination with selectivity data for BI 2536 and related compounds, illustrates important features for potency and selectivity. In particular, we show that the methoxy group of BI 2536 is an important specificity determinant against non-Polo-like kinases by taking advantage of a small pocket generated by Leu 132 in the hinge region of Polo-like kinase 1. The work presented here provides a framework for structure-based drug design of Polo-like kinase 1-specific inhibitors.

Key words: kinase selectivity, Plk1, Polo-like kinase, structure-based drug design

Abbreviations: ABL, Abelson tyrosine kinase; AKT, protein kinase B; AMPPNP, adenylimidodiphosphate; CDK1, cyclin-dependent kinase 1; CDK2, cyclin-dependent kinase 2; CHK1, checkpoint kinase 1; CK1α1, casein kinase 1 alpha 1; CK1δ, casein kinase 1 delta; CLK1, cell division cycle-like kinase 1; EGFR, epidermal growth factor receptor; FGFR1, fibroblast growth factor receptor 1; GSK3β, glycogen synthase kinase 3 beta; IRK, insulin receptor kinase; LCK, lymphocyte-specific protein kinase; LYN, v-yes-1 Yamaguchi sarcoma viral-related oncogene homologue; NEK2, nima-related kinase 2; PKA, protein kinase A; PKCβ, proten kinase C beta; Plk, polo-like kinase; SYK, spleen tyrosine kinase; TCEP, Tris(2-Carboxyethyl) phosphine.

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Members of the Polo-like kinase (Plk) family are important regulators of cell cycle progression (1,2). In mammals, four Plks have been identified: Plk1, Plk2 (Snk), Plk3 (Fnk or Prk) and Plk4 (Sak). All family members contain an N-terminal kinase domain and a C-terminal polo-box domain composed of either one (Plk4) or two polo-boxes, which is important for proper localization of Plks to their targets. Among different isoforms, Plk1 is best characterized and has emerged as one of the key regulators of mitosis, with important roles in the G2/M transition and throughout mitosis (3,4). The many processes in which Plk1 has been implicated include centrosome maturation, bipolar spindle formation, activation of Cyclin B/Cdk1, chromosome alignment, the spindle assembly checkpoint, chromatid separation, activation of the anaphase promoting complex/cyclosome and functions in mitotic exit and cytokinesis.

Compared to Plk1, less is known about the functions of other Plk isoforms. Plk2 acts during the G1/S transition and has been shown to be involved in centriole duplication (5). However, Plk2 function in the cell cycle seems non-essential, as knock-out mice are viable, albeit exhibiting retarded growth as well as reduced cell proliferation of cultured fibroblasts (6). Plk2 expression is induced by DNA-damaging agents, suggesting a role of this isoform in the DNA damage checkpoint (7,8). Plk3 has been proposed to have a biological function opposing that of Plk1 (2), but a recent report implicates Plk3 in S-phase entry and shows that Plk3 depletion prevents cell proliferation (9). Plk4 is the least conserved member of the Plk family. Plk4 is essential for cell viability, and has been implicated in cytokinesis (10,11), and centriole biogenesis and amplification (12,13).

Plk1 is highly expressed in proliferating cells and many cancers (14–20), and downregulation of Plk1 activity has been shown to inhibit cell proliferation in cancer cell lines and tumor xenografts (21–25). As such, Plk1 is a promising target for the development of anticancer drugs, and several companies have reported on efforts in this area (26–31). One particularly promising compound, BI 2536, has been shown to inhibit Plk isoforms *in vitro* with low nanomolar potency and with high selectivity against a large panel of kinases (32,33). BI 2536 potently inhibits the proliferation of a wide variety of human cancer cell lines, causes regression of tumor xenografts in mice, and is currently undergoing clinical trials (34).

We have previously published the structure of Plk1 kinase domain in complex with an Aurora A inhibitor (35) and hypothesized on residues important for specificity of Plk1 inhibitors. Since BI 2536 is the most potent and selective Plk inhibitor reported to date and is well characterized with respect to its biological activity, we have decided to determine the co-crystal structure of this compound with the Plk1 domain in order to add to our understanding of this clinical candidate. The structure, along with activity data for BI 2536 and

related compounds against a panel of kinases, confirms the importance of several active site residues that enable specific inhibition of Plk isoforms over other kinases. The work presented here provides a framework for the structure-based design of Plk1-specific inhibitors.

Materials and Methods

Crystallization

The T210V mutant kinase domain of human Plk1 was purified and crystallized as reported previously (35). Briefly, protein at 12 mg/mL in 50 mM HEPES, pH 7.5, 5 mM TCEP was incubated on ice with 5 mM BI 2536 (1:20 dilution of 100 mM stock in DMSO). BI 2536 was synthesized as described (36). One microlitre of the protein/inhibitor complex was combined with 1 μL of a reservoir solution consisting of 15% PEG 4000, 350 mM sodium malonate and 0.3 mM zinc acetate, and incubated for 5 days in a hanging drop over 500 μL of reservoir solution. Following a quick incubation (1–2 min) in a cryoprotecting solution consisting of reservoir solution with the addition of 25% glycerol, crystals were flash frozen in liquid nitrogen.

Data Collection and Structure Determination

Data were collected at the Advanced Photon Source (Argonne National Laboratories) at the IMCA-CAT Beamline 17-ID and were scaled and integrated using HKL2000 (37). The structure was solved by refining against the previously solved structure of Plk1 (2OWB) with coordinates for the waters and ligand removed. Model building proceeded with successive iterations of TLS refinement with Refmac 5 (38) and manual rebuilding with the program Coot (39). All data and statistics are presented in Table 1. Atomic coordinates have been deposited in the Protein Data Bank as entry 2RKU.

Enzymatic Activity Assays

Percent inhibition and IC50 values were determined with ATP concentrations at apparent K_m using the Invitrogen SelectScreen Kinase Profiling Service (Invitrogen Corporation, Carlsbad, CA, USA).

Results and Discussion

The structure of Plk1 in complex with BI 2536 is very similar to that reported previously for the AMPPNP and PHA-680626 complexes (35). The RMSD for 294 equivalent alpha carbons between the PHA-680626 and BI 2636 structures is 0.338 Å, with only a few minor side chain adjustments in the active site. The protein adopts the characteristic bilobal fold, with the inhibitor bound in a cleft between the N- and C-terminal lobes of the kinase domain. Figure 1 shows the binding mode of BI 2536 in the Plk1 active site. The inhibitor binds to the hinge region via two hydrogen bonds from the aminopyrimidine portion of the core to the backbone NH and carbonyl of Cys 133. This places the pteridinone moiety and the cyclopentyl group in the adenine and ribose portion of the ATP pocket, respectively, with the piperidine group pointing into the solvent.

Table 1: Data collection and refinement statistics

Data Collection	
Space group	$P3_221$
Unit cell parameters (Å)	$a = b = 66.6$, $c = 154.1$
Resolution (Å)	1.95
Redundancy[a]	6.6 (6.0)
Completeness (%)[a]	99.9 (99.9)
R_{merge}[a,b]	0.069 (0.505)
Average I/σ[a]	27.0 (3.6)
Refinement	
Number of reflections	27922
Number of test reflections	1494
R factor[c]	0.190
Free R Factor	0.241
RMS deviations	
Bond lengths (Å)	0.014
Bond angles (degree)	1.45
Average *B*-factors (Å^2)	
All atoms	45.0
Protein (no. non-H atoms)	44.2 (2411)
Inhibitor (no. non-H atoms)	31.7 (38)
Water (no. atoms)	50.1 (285)
Ramachandran plot statistics	
% in most favored region	89.5
% additional allowed region	9.3
% gerenous allowed region	1.2

[a]Values in parentheses refer to the highest resolution shell.
[b]$R_{merge} = \Sigma \, || - \langle I \rangle | \, / \, \Sigma \langle I \rangle$.
[c]R factor = $\Sigma || F_{obs}| - |F_{calc} || / \Sigma |F_{obs}|$ where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. Free R factor is calculated from a subset of reflections (5%) not used for refinement.

Three areas of the active site are exploited by BI 2536 to achieve the observed potency and selectivity. One of these extends along the hinge to the solvent-exposed N-methyl-piperidine. The phenyl ring of BI 2536 is sandwiched between the side chain of Leu 59 from the top (N-lobe) and the side chain of Arg 136 from the bottom (C-lobe). The methoxy group is accommodated in a pocket created by Leu 132 of the hinge region. Both Arg 136 and Leu 132 are relatively specific for PLK1, with the former being frequently a glycine in other kinases and the latter more commonly a bulky tyrosine or phenylalanine (see Table 2 for a listing of key active site residues in Plk1 and their counterparts in other kinases). The presence of larger side chains in place of Leu 132 is predicted to clash with the methoxy group of BI 2536 and hence provides an opportunity for selectivity, as has been suggested in the case of NPM-ALK (40). The amide linker of the inhibitor mediates a network of interactions that involve residues from both the N- and C-lobes of Plk1, with the NH and carbonyl forming hydrogen bonds with the main chain carbonyl of Leu 59 of the glycine-rich loop and the side chain of Arg 57, respectively. The side chain of Arg 136, stabilized by water-mediated hydrogen bonds with the side chain of Glu 140 and the main chain carbonyl of Leu 59 (not shown in Figure 1), interacts with the amide linker as well as the piperidine through van der Waals contacts, providing additional binding affinity through this unique reside.

Another important area for potency and selectivity of BI 2536 is at the roof of the binding site. Here, the ethyl group is accommodated

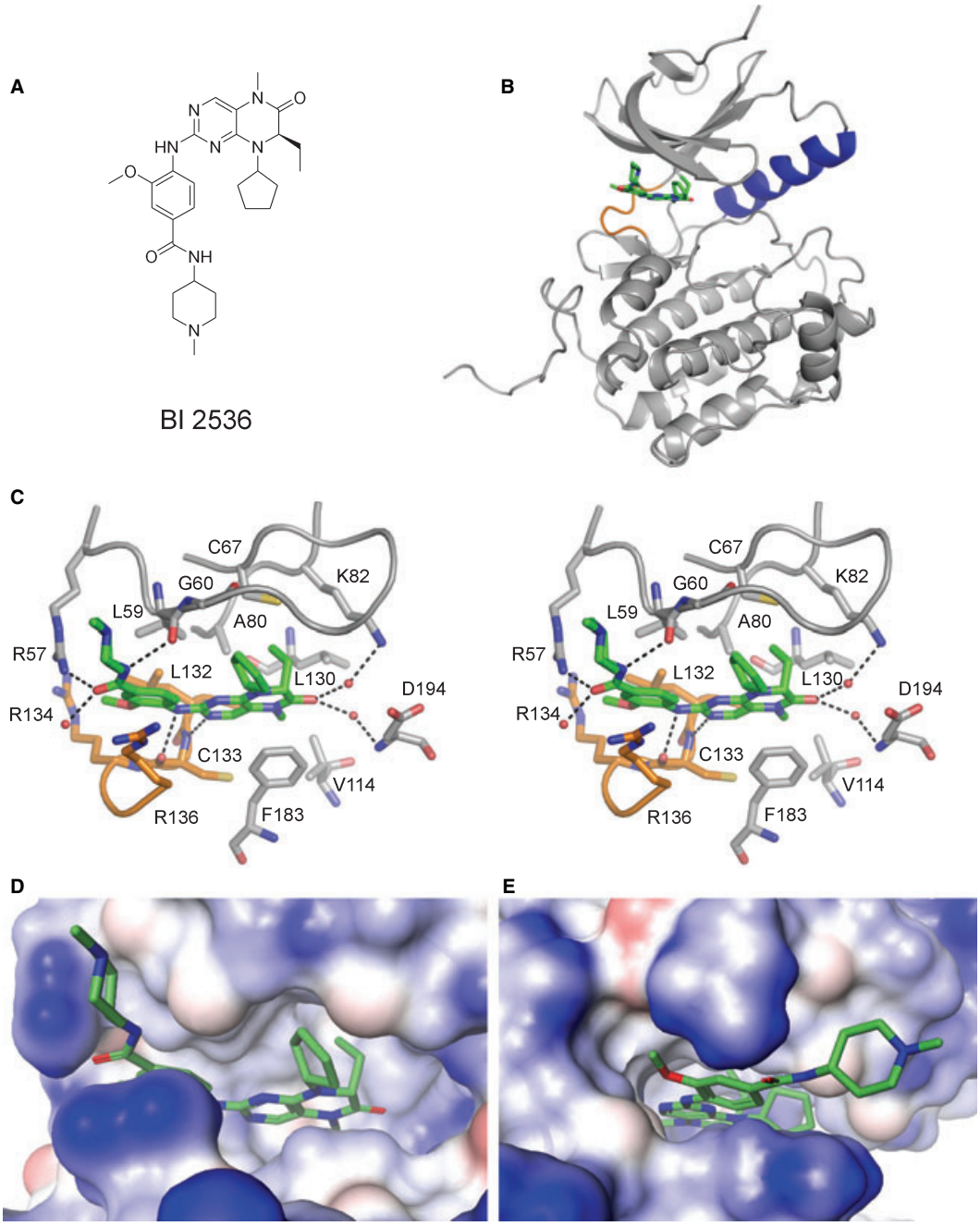


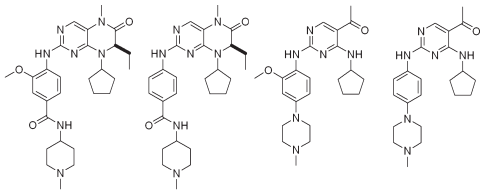
Figure 1: Binding mode of BI 2536 in Plk1. (A) Chemical structure of BI 2536. (B) Overview of the kinase domain with BI 2536 bound to the active site (green sticks). Helix alpha C is shown in blue and the hinge region in orange. (C) Divergent stereo view of the binding mode of BI 2536. (D, E) Surface representations of BI 2536 in the Plk1 active site (colored by electrostatic potential) showing the placement of the cyclopentyl and ethyl groups in the roof of the active site (D), and of the methoxy group in the hinge region (E). Panels B and C were generated using Pymol (http://www.pymol.org), panels D and E, including electrostatic surface calculation, were generated using proprietary molecular visualization software.

in a small pocket formed by Cys 67, Lys 82, Ala 80 and Leu 130, and the cyclopentyl is positioned near Leu 59, Gly 60 and Cys 67. The side chain of Cys 67 is rotated relative to the position in the previously published structure in order to accommodate the ethyl group. This residue is more commonly a valine in other kinases, and this variation will likely interfere with the placement of one or both of the substituents near the roof of the binding site. The presence of Phe 183 (commonly a leucine or methionine in other kinases) at the bottom of the binding site further enhances binding affinity through π–π stacking with the pteridinone moiety.

Table 2: Comparison of selected active site residues in Plk isoforms and other kinases[a]

	R^{57}	C^{67}	A^{80}	V^{114}	L^{130}	L^{132}	R^{136}	F^{183}
Plk1	**R**	**C**	**A**	**V**	**L**	Y	**R**	**F**
Plk2	K	**C**	**A**	**V**	**L**	Y	**R**	**F**
Plk3	**R**	**C**	**A**	**V**	**L**	**L**	K	**F**
Plk4	N	V	**A**	L	**L**	M	G	L
SYK	K	V	**A**	**V**	M	M	G	L
EGFR	K	V	**A**	C	T	**L**	G	L
NEK2	Y	**C**	V	**V**	M	Y	G	**F**
ABL	H	V	**A**	**V**	T	F	G	L
CLK1	D	V	**A**	**V**	F	**L**	L	L
LCK	E	V	**A**	V	T	Y	G	L
Aurora A	**R**	V	**A**	L	**L**	Y	G	L
IRK	**R**	V	**A**	**V**	M	**L**	G	M
CK1δ	**R**	I	**A**	P	M	**L**	P	L
PKA	K	V	**A**	**V**	M	Y	G	L
CDK2	E	V	**A**	**V**	F	F	Q	L
PKCβ	M	V	**A**	T	M	Y	G	M
LYN	K	V	**A**	**V**	T	Y	G	L
CK1α1	**R**	I	**A**	P	M	**L**	P	L
CDK1	V	V	**A**	**V**	F	F	M	L
AKT	K	V	**A**	T	M	Y	G	M
CHK1	Q	V	**A**	**V**	**L**	Y	G	L
GSK3β	K	V	**A**	**V**	**L**	Y	E	L
FGFR1	K	V	**A**	I	V	Y	G	L

[a]Kinases other than Plk isoforms are listed in order of decreasing potency of BI 2536 (see Figure 2). Residues identical to those in Plk1 are bolded. Residue identities were determined from known crystal structures or by alignment with sequences with known structures.



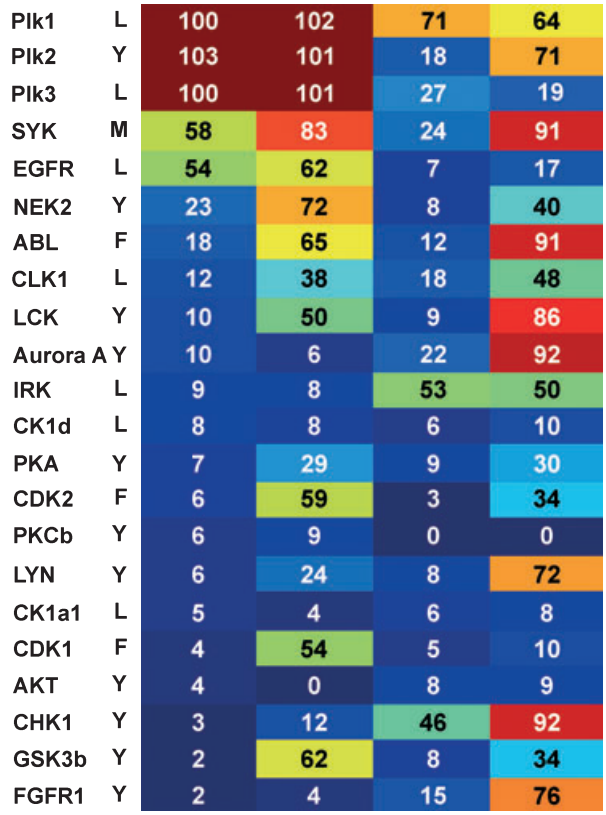


Figure 2: Inhibition profile of BI2536 and related inhibitors against a panel of kinases. Percent inhibition values (1 μM, average of three measurements) are shown; fields are color coded in decreasing potency from red to blue. The residue equivalent to Leu 132 in the hinge region of Plk1 is shown for each kinase. Kinases are listed in the same order as in Table 2.

The third area exploited by BI 2536 lies towards the back of the binding site, where two water-mediated hydrogen bonds are formed from the pteridinone carbonyl to the side chain of Lys 82 and the backbone NH of Asp 194, respectively, and where the methyl group is buried in a pocket near Val 114, Phe 183 and the gatekeeper residue Leu 130. These interactions will contribute significantly to the potency of BI 2536 but are probably less important for selectivity than interactions at the hinge and roof of the pocket, where larger substituents are exploiting less conserved features of the active site. Overall, the structure reveals an inhibitor that is exquisitely matched to the binding site presented by Plk1 and that benefits from many specific interactions that together allow for the observed potency of BI 2536 against this kinase.

In order to better understand the contributions of the various active site regions to potency and selectivity of Plk-specific inhibitors, we determined the inhibition profile of BI 2536 (**1a**) and three related compounds against a panel of kinases (Figure 2). We confirmed the reported high degree of specificity of BI 2536 (32), with complete inhibition at 1 μM observed for Plk1, Plk2 and Plk3 and approximately 50% inhibition of SYK and EGFR. Examination of Table 2 and Figure 2 shows that, with the exception of Plk2 (which will be discussed below), none of the kinases with a bulky residue at the position of Leu 132 in Plk1 were inhibited by BI 2536 to any appreciable degree. This suggests that the size of the side chain at this position is an important determinant for sensitivity towards BI 2536. Consistent with this hypothesis, **1b**, an analog of BI 2536 lacking the methoxy group, exhibits a more promiscuous inhibition profile (Figure 2), with potencies for six kinases (NEK2, ABL, LCK, CDK2, CDK1 and GSK3β) changed from negligible inhibition by **1a** to 50% or more. All of these six kinases have a tyrosine or phenylalanine in the position of Leu 132 in Plk1, demonstrating that the methoxy group allows BI 2536 to discriminate against kinases with a bulky residue at this position. It is interesting to note that only modest changes in potency were observed for any of the kinases with leucine at this selectivity site, indicating that a methoxy group at this location does not contribute significantly to the binding energy for BI 2536, but rather serves as a selectivity determinant. The use of a methoxy group to gain selectivity based on the residue identity at position Leu 132 in Plk1 has been reported for other kinases, for example NPM-ALK (40), demonstrating that this approach might be useful to address selectivity concerns in other targets.

The importance of the methoxy group was further confirmed by data obtained on two diaminopyrimidine compounds, **2a** and **2b**, which share some features with BI 2536 and are predicted to bind to the kinase active site in a similar orientation. Compound **2a**, containing a methoxy group in a position equivalent to that in BI 2536, displayed a fairly clean inhibition profile, with only Plk1 and IRK inhibited at more than 50% at 1 μM. Compound **2b**, which lacks the methoxy group, was able to inhibit many more kinases in our panel, with eight additional kinases inhibited at 50% or more. As was the case for **1b**, all of these additionally inhibited kinases have a bulky residue at the position of Leu 132 in Plk1 that would prevent **2a** from binding. Again, the lack of methoxy group had only a marginal effect on the inhibition of kinases with leucine at the selectivity site, confirming that this group contributes little to potency. We observed that **2b** inhibits a larger number of kinases to a larger degree than **1b**. One likely reason for this is that lack of a bicyclic structure in the core allows for more flexibility in the placement of the cyclopentyl and carbonyl groups, thereby allowing **2b** to better accommodate active sites of a larger variety of kinases, in particular kinases (such as Aurora A) with less bulky residues at the position equivalent to Phe 183 of Plk1 (35). In addition, the lack of an ethyl group in **2a** and **2b** is expected to more readily allow binding to kinases with valine in place of Cys 67 in Plk1. The inhibition profile of **2b**, therefore, highlights the contributions of residues away from the hinge region, such as Cys 67, Phe 183 and Val 114 to Plk1 inhibitor affinity and specificity.

An interesting feature of the inhibition profile of BI 2536 is that, while selectivity against kinases other than Plk isoforms is strong, the compound does not discriminate between Plk1, Plk2 and Plk3. In order to more closely examine selectivity within the Plk family, we determined IC50 values for BI 2536 (**1a**), as well as compounds **1b**, **2a** and **2b** against Plk1, Plk2 and Plk3 (Table 3). The data for BI 2536 are consistent with previously published reports (32,41) and confirm that there is essentially no difference in the potency of BI 2536 against the three isoforms tested, with inhibition observed at low nanomolar concentrations for all three.

It is interesting to note that removal of the methoxy group in **1b** had no effect on the measured IC50 values. We speculate two possible explanations for this, especially with regard to Plk1 and Plk3, which both have a leucine residue in the hinge region. First, any potency gain from partial burying of the methyl group might be offset by the desolvation penalty incurred from placing oxygen into a hydrophobic environment. Second, the lack of a methoxy group in **1b** might allow for slight changes in the binding mode resulting in improved interactions, for example a more ideal placement of the aminopyrimidine group for hydrogen bonding with the hinge region. In fact, such a shift in binding mode was observed when **1b** was docked into the active site of the Plk1 **1a** structure (data not shown), indicating that the interaction of **1a** with the Plk1 hinge donor-acceptor pair might not be optimal.

Table 3: IC50 values of BI 2536 and related inhibitors against Plk isoforms[a]

	IC 50 (nM)			
	BI 2536 (**1a**)	**1b**	**2a**	**2b**
Plk1	8.0 ± 2.9	5.0 ± 2.4	145.3 ± 68	345.3 ± 124
Plk2	16.8 ± 7.4	11.5 ± 4.9	>10.000[b]	367.3 ± 41.5
Plk3	13.6 ± 7.3	6.7 ± 2.2	2583.3 ± 609	3490 ± 1300

[a]Values represent the average of three determinations with standard deviations shown.
[b]Two of the three readings for this value were out of the detection range of the assay, while the third reading was 8 000 nM.

Except for the change of Plk1 Leu 132 to tyrosine in Plk2, the active sites of Plk1, Plk2 and Plk3 are very similar (Table 2), and this explains the observed lack of selectivity, especially when comparing Plk1 to Plk3. The activity of **1a** against Plk2 is surprising, because the methoxy group of the compound is expected to clash with tyrosine in the Plk2 hinge region. We speculate that **1a** and **1b** both have a good overall fit with the Plk2 active site (as evidenced by their low IC50 values), and this allows the methoxy group in **1a** to be accommodated in Plk2 through readjustments of compound and/or protein in the hinge region or elsewhere in the binding pocket, possibly resulting in an improved fit relative to **1b** and thus compensating for the loss of affinity at the hinge region. A similar adjustment might not be possible in kinases that bind **1a** with lower affinity, or for compounds that bind to Plks with lower affinity, such as **2a**. Consistent with this speculation, inhibition of Plk2 by **2a**, a less potent Plk1 inhibitor, was sensitive to the presence of the methoxy group. While the compound lacking the methoxy was equipotent against Plk1 and Plk2 (345 and 367 nM, respectively), addition of the methoxy group in **2b** completely eliminated the Plk2 activity, while having a negligible effect on Plk1 potency. A structure of Plk2 with BI 2536 might provide insight to the inhibition mechanism of Plk2 by BI 2536 as well as constitute part of a selectivity platform for Plk isoform-specific inhibitors.

As outlined in the introduction, the functions of other Plk isoforms are only beginning to be understood in detail. Therefore, isoform-specific Plk inhibitors might be necessary to avoid off-target effects, and would provide valuable tools to further elucidate Plk biology. The active site of Plk4 is quite distinct from that of other isoforms, and selectivity for this isoform will likely not be challenging to obtain. In support of this, Johnson *et al.* (41) recently reported that Plk4 displays a distinct pharmacological profile and is the only Plk isoform that is not inhibited by BI 2536. For other isoforms, apart from the leucine to tyrosine change in the hinge region of Plk2 discussed above, there are no significant changes in active site residues that we identified as important for specificity of BI 2536. Therefore, the design of selective and potent compounds for these isoforms is likely to be more challenging. Our data on the modestly potent compounds, **2a** and **2b**, demonstrate that it is possible to exploit the difference at Plk1 Leu 132 in order to achieve selectivity against Plk2, and these compounds also show some discrimination against Plk3 (Figure 2 and Table 3). For the design of next generation Plk inhibitors, an approach that attempts to optimize the interaction with the donor–acceptor pair in the hinge region, while still exploiting the leucine pocket in Plk1 and Plk3, might be a promising path to increase potency while maintaining or improving isoform specificity. The data presented here contribute to a structure-based drug design platform to address these challenges.

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