Mitotic arrest and slippage induced by pharmacological inhibition of Polo-like kinase 1

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ABSTRACT

Exposure to drugs that interfere with microtubule dynamics block cell cycle progression at mitosis by prolonged activation of the spindle assembly checkpoint (SAC). Cells can evade mitotic arrest and proceed to interphase without chromosome segregation by a process termed mitotic slippage that involves Cyclin B1 degradation without checkpoint inactivation. Here, we explored the cellular response to small-molecule inhibitors of Polo-like kinase 1 (Plk1), an important regulator of cell division. We found that the clinical Plk1 inhibitors BI 2536 and BI 6727, both unexpectedly, induced a dose-dependent cellular drug response: While mitotic arrest was induced in cancer cell lines and primary non-transformed cells across the entire range of concentrations tested, only high concentrations seemed to promote mitotic slippage. Since this observation contrasts with the effects expected from studies reporting RNAi-mediated Plk1 depletion in cancer cells, we wondered whether both ATP-competitive inhibitors target unknown kinases that are involved in signaling from the spindle assembly checkpoint (SAC) and might contribute to the mitotic slippage. A chemical proteomics approach used to profile the selectivity of both inhibitors revealed that SAC kinases are not targeted directly. Still, the activities of Cdk1/Cyclin B1 and Aurora B, which plays important roles in the error correction of false microtubule-kinetochore attachments and in checkpoint signaling, were shown to be downregulated at high inhibitor concentrations. Our data suggest that the inhibition of Plk1 activity below a certain threshold influences Aurora B activity via reduced phosphorylation of Fox M1 and Survivin leading to diminished levels of Aurora B protein and alteration of its subcellular localization. Within the spectrum of SAC proteins that are degraded during mitotic slippage, the degradation of Cyclin B1 and the downregulation of Aurora B activity by Plk1 inhibition seem to be critical promoters of mitotic slippage. The results...
1. Introduction

The polo-like family of serine/threonine kinases plays a crucial role in the regulation of cell cycle progression (Archambault and Glover, 2009; Barr et al., 2004; van de Weerd and Medema, 2006). Because cancer cells require Plk1 for survival (Liu and Erikson, 2003; Spankuch-Schmitt et al., 2002b), Plk1 has been investigated intensively as a target for novel anti-cancer agents (Liu et al., 2011; McInnes et al., 2006; Strebhardt, 2010). Potent small-molecule inhibitors of mammalian Plk1, BI 2536 and BI 6727, which inhibit Plk1 enzyme activity at low nanomolar concentrations, cause mitotic arrest, induce apoptosis in human cancer cell lines of different origin and are currently evaluated in multiple clinical cancer trials (Lenart et al., 2007; Rudolph et al., 2009). Antimitotic agents like Plk1 inhibitors or compounds that interfere with microtubule dynamics, including taxanes and vinca alkaloids induce the spindle assembly checkpoint (SAC) that prevents the progression to anaphase until all the chromosomes are properly attached to kinetochores and are under the right tension (Manchado et al., 2012; Musacchio and Salmon, 2007). The anaphase-promoting complex/cyclosome (APC/C) is an E3-ubiquitin ligase that triggers mitotic exit during normal mitotic progression mostly by targeting Cyclin B1 for degradation, thus inhibiting Cdk1 activity. The SAC targets CDC20, a co-factor of the APC/C. In particular, by keeping CDC20 in check the APC/C remains inactive which prevents the destruction of two key substrates, Cyclin B1 and Securin. However, long-term activation of the SAC during exposure to antimitotic agents may cause the levels of proteins essential to maintain mitotic arrest to fall, triggering mitotic slippage, which occurs when cells exit mitosis, without chromosome segregation or cell division (Andressen et al., 1996; Brito and Rieder, 2006). Spontaneous mitotic slippage takes place through slow ubiquitylation of Cyclin B1 by APC/C and subsequent proteasome-dependent degradation despite mitotic checkpoint activity. The core components of the SAC include MAD2, BUB1/Mad3, BUB3, MAD1 and the kinases BUB1, multipolar spindle -1 (MPS1) and Aurora B. Moreover, Aurora B is part of the chromosomal passenger complex (CPC), which comprises INCENP, Borealin, Survivin and Aurora B (Musacchio and Salmon, 2007). The CPC, which can be found at chromosomes and kinetochores in early mitosis, controls the attachment of microtubules to chromosomes, sister chromatid cohesion and the SAC. The use of the Aurora B-specific inhibitors, like Basertib, stimulates mitotic slippage in different types of cancer cells (Marxer et al., 2013; Yang et al., 2007).

Comprehensive investigations of cell populations and different cell types have revealed that cancer cells display profound intra- and interline variation following prolonged exposure to antimitotic drugs encompassing death during mitosis, mitotic slippage and death in the subsequent interphases, mitotic slippage and cell survival, or no mitotic entry (Gascoigne and Taylor, 2008; Marxer et al., 2013; Sakurikar et al., 2012). The determination of the type of cellular response to be expected upon treatment with a specific antimitotic agent and the study of factors that determine the outcome is important for the identification of predictive factors that determine the patients’ response and thus for a tailored and rational design of clinical trials testing efficacy of novel anti-cancer agents.

In this study we examined the dose-dependent response of cancer cells and primary human cells to the clinical Plk1 inhibitors BI 2536 and BI 6727. While in the entire range of concentrations tested both inhibitors induced mitotic arrest, at higher concentrations mitotic slippage was observed. Our study revealed that activities of Cdk1/Cyclin B1 and Aurora B are inhibited at higher drug concentrations accompanied by reduced protein levels and dyslocalized Aurora B suggesting that reduced levels of Aurora B activity and Cyclin B1 contribute to mitotic slippage of human cells upon exposure to Plk1 inhibitors.

2. Materials and methods

2.1. Preparation of primary cells and cell culture

HUVEC and fibroblasts were isolated and cultured as described (Raab et al., 2011) Keratinocytes were grown in Dermalife® K Medium Complete Kit (Cell Systems). Immortalized human retinal pigment epithelial cells (hTERT-RPE1) expressing Plk1wt or Plk1as were propagated as described (Burkard et al., 2012). Cancer lines were purchased from the American Type Culture Collection, cultured with 10% FCS (PAAl) at 37°C with 5% CO2 in a humidified atmosphere: MEM (Sigma) for HeLa, RPMI 1640 (PAAl) for HL-60. MDA-MB-468 cells were grown in Leibovitz’s L-15 (PAAl) without CO2. For Plk1 inhibition, cells were treated with 10–2000 nM BI 2536 or BI 6727 (ICS).

Bone marrow samples were obtained from AML patients at diagnosis or according to study schedule. All patients gave written informed consent and collection of samples was approved by the ethics committee of the Goethe-University. Cells were collected with heparinized syringes and purified on a Ficoll density gradient. For long-term cultures (LTC)-AML (FFM5, FFM12), mononuclear cells were isolated as described (Rossmanith et al., 2001). AML cells were maintained in X-Vivo 10 (Lonza)/10% FCS HyClone (Perbio)/1% l-glutamine (Sigma) and cytokines hIL-3 (20 ng/ml), hTPO
Figure 1 – Dose-dependent effects of Plk1 inhibition on HeLa cells. (A) Representative examples of the cell cycle status monitored by FACS after 48 h and inhibitor concentrations are shown. Mitotic indices of BI 2536-treated HeLa cells. Columns, mean of three independent experiments; bars, SE. (B) Western blot analysis of cells treated for 24 h with the indicated inhibitor concentrations. Cell lysates were immunoblotted for Plk1, Phospho-Aurora B (T232)/Aurora C (T198), Aurora B, histone H3 phosphorylated at S10 (pH3), Cdk1, Cyclin B1, Securin, Phospho-Bcl-XL, Induced myeloid leukemia cell differentiation protein Mcl-1, MPM-2 and β-Actin. (C) Cells treated for 24 h with 1 μM BI 2536 or BI 6727 were labeled using antibodies for Pericentrin, α-Tubulin and 4,6-diamidino-2-phenylindole (DAPI) for analysis by immunofluorescence microscopy.
(25 ng/ml), hSCF (50 ng/ml) and hFlt3-ligand (50 ng/ml) (all Peprotech).

The three-dimensional (3-D) colony formation assay was performed as published (Heiligans et al., 2012).

2.2. Transfections

For DNA transfection, the X-tremeGene 9 DNA transfection reagent (Roche) was used, according to the manufacturer’s protocol.

2.3. Cell cycle, apoptosis and cell proliferation assays

Nocodazole treatment (Sigma) was conducted as described (Yuan et al., 2002). For cell cycle analysis, cells were harvested, washed, fixed and stained as described (Raab et al., 2011). Cell cycle quantification was performed using a FACS Calibur instrument and Cellquest Pro software (both BD Biosciences). The activity of caspase-3/7 was determined by Caspase-Glo®3/7 Assay, according to the manufacturer’s instructions (Promega, Madison, WI, USA). Apoptotic loss of membrane asymmetry was analyzed by staining for PE Annexin V and 7-AAD (BD Biosciences) on a FACS Calibur instrument. Cell viability and proliferation assays were conducted using the Cell Titer-Blue® Cell Viability Assay (Promega), as described (Raab et al., 2011).

2.4. Antibodies

Antibodies were used at the following concentrations: mouse monoclonal anti-Plk1 (1:1000), Cdk1 (1:1000), Cyclin B1 (1:5000), Bcl-XL (1:1000), Phospho-Aurora A,B,C (1:1000), Phospho-Histone bodies, Abcam), Survivin (1:1000), Mcl-1 (1:1000) (Enzo), Aurora a (Raab et al., 2011).

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2.5. Kinase assays

Immunoprecipitation assays using Aurora B-, Cdk1- or Plk1-specific antibodies were performed as described (Matthes et al., 2010). To measure kinase activities of Plk1 and Aurora B (Biornol), in vitro kinase assays were performed as described (Matthes et al., 2010) in the presence of Histone H1/3 (New England Biolabs) (Cohen et al., 1997; Wang et al., 2007).

2.6. Statistical methods

All experiments were performed at least in triplicate. Standardization and statistics were determined as described (Spankuch-Schmitt et al., 2002a). Significant differences (p < 0.05) are indicated in the figures with an asterisk.

3. Results

3.1. Pharmacological inhibition of Plk1 in cancer cells promotes mitotic exit delay and slippage

Asynchronously-growing HeLa cervical cancer cells were treated with serial dilutions of the Plk1 inhibitors BI 2536 or BI 6727 to examine the effect of Plk1 inhibition on mitotic progression. First, we assessed the effect of BI 2536 on the cell cycle distribution by FACS and western blot analysis. Rising concentrations (10–2000 nM) caused a pronounced enrichment of cells with a 4N DNA content that was indicative of a G2/M arrest (Figure 1A, left panel). Microscopic examination revealed that the proportion of mitotic cells (mitotic index) increased until 100 nM BI 2536 of treatment and, rapidly decreased at higher concentrations (Figure 1A, right panel). Some cells treated with high concentrations of BI 2536 underwent chromosome decondensation and formation of nuclear envelopes without the normal processes of mitosis, such as chromosome segregation and cytokinesis. This process was defined as ‘mitotic slippage’. Cells escape from mitotic arrest induced by BI 2536 and subsequently enter the G1 phase without cell division. At concentrations up to ~100 nM a strong accumulation of Plk1, Aurora B, Cyclin B1 (the regulatory subunit of the cyclin-dependent kinase 1) and Securin (a protein involved in control of the metaphase–anaphase transition) was observed (Figure 1B). Remarkably, at BI 2536 concentrations > ~100–500 nM declining levels of Plk1, Aurora B, Cyclin B1 and Securin were observed accompanied by descending SAC activity indicated by MPM-2 staining when compared to those at 50 nM doses (Figure 1B). This range of higher concentrations was associated with a repeated round of DNA synthesis as indicated by an increase of the >4N population (Figure 1A) suggesting that BI 2536 could induce mitotic slippage or failure of cytokinesis. The microscopic inspection of HeLa cells revealed that 1 μM BI 2536 increased the level of multinucleated cells, confirming perturbed mitotic progression at high drug concentrations (Figure 1C). Antibodies, that recognize Pericentrin, which is an integral component of the centrosome and serves as a multifunctional scaffold for anchoring numerous proteins, were used to label centrosomes in treated cells (Figure 1C). Interestingly, mitosis associated with slippage at higher BI 2536 concentrations (~500 nM) seemed to increase the overall cell number within...
the observation period of 72 h compared to 50 nM (Figure 1D). To analyze the effect of Plk1 inhibition on tumor cell survival in a more physiological cell culture model, we applied a three-dimensional colony formation assay (Hehlgans et al., 2012, 2009). Treatment of HeLa cells with BI 2536 resulted in decreased cellular survival already at a concentration of 10 nM and further decrease at 100 nM when compared to control-treated cells (Figure 1E). Importantly, treatment of cells with higher concentrations increased the surviving fractions again with a local maximum at 1 μM BI 2536.
To consolidate the link between mitotic exit delay/slippage and Plk1 inhibition, we conducted similar experiments with another Plk1 inhibitor, called BI 6727. Once again, this drug induced a strong G2/M arrest (Figure 1A, middle panel) associated with high levels of Plk1, Aurora B, Cyclin B1 and Securin (Figure 1B). At somewhat higher concentrations (> ~500 nM) compared to BI 2536, levels of Plk1, Aurora B, Cyclin B1 and Securin descended again (Figure 1B) accompanied by an increase of the >4N population (Figure 1A). We could also demonstrate descending SAC activity by using MPM-2 antibodies in those cells (Figure 1B). The microscopic inspection corroborated that a BI 6727 concentration of 1 μM promotes an increase of the >4N population indicating that BI 6727 could also induce mitotic slippage or failure of cytokinesis (Figure 1C). Moreover, the treatment with BI 6727 concentrations of 1–2 μM increased the surviving fractions slightly compared to 100 nM (Figure 1D and E).

We next examined if other cancer lines also respond with mitotic arrest and slippage to increasing concentrations of BI 2536 and BI 6727. Across the entire range of concentrations tested MDA-MD-468 breast cancer cells were enriched in G2/M (Figure S1A). At BI 2536 and BI 6727 concentrations > ~500 nM levels of Plk1, Aurora B, Cyclin B1, Securin and pH3 decreased (Figure S1B). The microscopical inspection demonstrated also higher levels of multinucleated cells at 1 μM BI 2536 compared to the treatment with 100 nM BI 2536 (Figure S1C). Concentrations >10 nM BI 2536 and >50 nM BI 6727, respectively led to a slight increase in cell numbers (Figure S1D). Furthermore, treatment of HL-60 cells with BI 2536 resulted in decreased cellular survival already at a concentration of 10 nM and further decrease at 100 nM when compared to control-treated cells (Figure S1E). Importantly, treatment of cells with higher concentrations increased the surviving fractions again with a local maximum at 1 μM BI 2536. Collectively, these results confirm that robust inhibition of Plk1 causes mitotic exit delay and slippage, respectively.

### 3.2. Pharmacological inhibition of Plk1 in primary non-cancer cells promotes mitotic exit delay and slippage

Intensive research has focused on the responses of cancer cells to various inhibitors of mitosis and it is known that apoptosis is a common outcome triggered either by p53-dependent or 53-independent pathways (Upreti et al., 2006; Yamaguchi et al., 2004). However, the response of primary non-cancer cells to mitotic inhibitors is less well understood. The treatment of Human Umbilical Vein Endothelial Cells (HUVEC) with increasing concentrations of BI 2536 caused a strong G2/M arrest (Figure 2A). At concentrations up to ~100 nM levels of Plk1, Aurora B, Cyclin B1, Securin and pH3 decreased (Figure 2B). The microscopical inspection demonstrated also higher levels of multinucleated cells at 1 μM BI 2536 compared to the treatment with 100 nM BI 2536 (Figure S1C). Concentrations >10 nM BI 2536 and >50 nM BI 6727, respectively led to a slight increase in cell numbers (Figure S1D). Furthermore, treatment of HL-60 cells with BI 2536 resulted in decreased cellular survival already at a concentration of 10 nM and further decrease at 100 nM when compared to control-treated cells (Figure S1E). Importantly, treatment of cells with higher concentrations increased the surviving fractions again with a local maximum at 1 μM BI 2536. Collectively, these results confirm that robust inhibition of Plk1 causes mitotic exit delay and slippage, respectively.

### Figure 3

**Determination of Plk1 and Aurora B activities in vitro and in vivo following inhibitor treatment.** (A) Representative in vitro kinase assay monitoring the autophosphorylation activity of Plk1 and Aurora B at increasing concentrations of BI 2536 or BI 6727. (B) Cancer cell lines (HeLa, MDA-MB-468, HL-60) and primary cells (HUVEC, Fibroblasts, Keratinocytes) were treated (24 h) with the indicated concentrations. Immunoprecipitated Plk1 or Aurora B was subjected to kinase assays using Histone H1/H3 as substrates.
Figure 4 — Dose-and time-dependent effects of Plk1 inhibition on the proliferation, cell cycle and apoptosis of hTERT-RPE1 cells expressing Plk1 wt or Plk1 as. (A) Representative examples of the cell cycle status monitored by FACS after 48 h and inhibitor concentrations are shown. (B) Lysates from Plk1 wt and Plk1 as cells following 24 h treatment with BI 2536 were immunoblotted for GFP-Plk1, Phospho-Aurora B (T232)/Aurora E, Cyclin B1 and β-Actin. (C) Plk1 wt and Plk1 as cells were treated for 24 h with BI 2536 at the indicated concentrations and analyzed for the expression of β-Actin. (D) Plk1 wt and Plk1 as cells were treated for 72 h with BI 2536 at the indicated concentrations and analyzed for the expression of β-Actin.
increased (Figure 2B). At concentrations > ~100–500 nM levels of Plk1, Aurora B, Cyclin B1 and Securin decreased when compared to those at 50–100 nM doses (Figure 2B), indicating that the drug response in HUVEC is also biphasic as previously seen in cancer cells. Moreover, Cdk1/Cyclin B1 precipitated from cell lysates and subjected to in vitro kinase assays showed a peak activity between 50 and 500 nM followed by a decline of activity at higher drug concentrations (Figure 2B, lower panels). Analyses by FACS and by microscopy showed that the treatment with BI 2536 induced an increase of the >4N population/multinucleated cells indicating an induction of mitotic slippage or failure of cytokinesis (Figure 2A,C). Whereas it has previously been shown that Plk1-specific siRNAs inhibited cancer cell proliferation while leaving normal proliferating cells largely unaffected (Liu et al., 2006; Raab et al., 2011; Spankuch-Schmitt et al., 2002a; Spankuch et al., 2004), BI 2536 had an anti-proliferative effect in both, cancer cells and normal primary cells (Figure 1D, Figure 2D). At higher BI 2536 concentrations > ~500 nM the decline of cell numbers was stopped (Figure 1D, Figure 2D). BI 6727 induced a similar biphasic response at somewhat higher concentrations compared to BI 2536 (Figure 2A–D). Additional primary, non-tumor cells (Fibroblasts, Keratinocytes) show a prominent mitotic arrest across the entire spectrum of tested concentrations (Figures S2A, S3A), respond to high Plk1 inhibitor concentrations (>500 nM) similarly with descending levels of Cyclin B1, pH3, Aurora B and Plk1 (Figures S2B, S3B) and show growth inhibition at increasing concentrations of both inhibitors (Figures S2C, S3C).

3.3. Phosphorylation of anti-apoptotic Bcl-2 proteins

The prevailing model suggests that the control of Cyclin B1 degradation and the activation of death signaling dictate cell fate following mitotic arrest. Evidence for the mutual regulation of both pathways came from studies showing Cdk1/Cyclin B1 inactivates anti-apoptotic members of the Bcl-2 protein family (Bcl-2, Bcl-X, and Mcl-1) by phosphorylation following treatment with anti-tubulin chemotherapeutics (Terrano et al., 2010; Wertz et al., 2011). We monitored the levels of Bcl-X, phosphorylation and Mcl-1, to evaluate the role of both anti-apoptotic proteins for cells treated with Plk1 inhibitors. The intensity of Bcl-X, phosphorylation paralleled the levels of Cyclin B1 in HeLa cells at increasing BI 2536 concentrations (Figure 1B, left panel). Levels of Cyclin B1 and Mcl-1 were reciprocal suggesting that high levels of Cdk1/Cyclin B1 activity lead to phosphorylation and subsequent degradation of Mcl-1 (Figure 1B, left panel). At high inhibitor concentrations >500 nM increasing levels of Mcl-1 and descending levels of phospho-Bcl-X, were observed indicating that the anti-apoptotic potential of both proteins increases. Moreover, the treatment of HeLa cells with BI 6727 (Figure 1B, right panel) and the treatment of MDA-MB-468 cells with BI 2536 or BI 6727 confirmed that high inhibitor concentrations increases levels of Mcl-1 and decreases levels of phospho-Bcl-X, (Figure S1B) supporting the model that cellular survival in culture is increased at drug concentrations between 500 and 2000 nM.

3.4. Analysis of SAC kinases by a kinome-wide chemical proteomics approach

Decreasing levels of mitotic proteins (Cyclin B1, Plk1, Aurora B) as drug concentrations increased and the appearance of multinucleated cells indicate that SAC deactivation and mitotic slippage may have occurred. As SAC signaling is regulated by multiple kinases, we tested the hypothesis by using a kinome-wide proteomics approach that the ATP-competitive Plk1 inhibitors BI 2536 and BI 6727 may target as yet unknown protein kinases thereby contributing to mitotic slippage. To this end, we have developed a chemical proteomics screening method that allows the investigation of multiple kinases under near-physiological conditions in parallel (Bantscheff et al., 2007). The approach contains two major components: Firstly, an affinity purification matrix termed Kinobeads which consists of seven immobilized nonselective kinase inhibitors. It allows the purification and identification of several hundred kinases from cells (Bantscheff et al., 2007). Secondly, an intensity-based label-free quantitative mass spectrometry (MS) that enables the identification and relative quantification of the purified proteins across many biological samples (Patel et al., 2009). This Kinobead competition assay was applied to the quantitative selectivity profiling of BI 2536 and BI 6727. Using MS, a total of 146 kinases were identified and quantified (Figure S4A). For the recruiting of SAC proteins to kinetochores and for generating robust SAC activity the protein kinases MPS1, BUB1, BUBR1, Plk1, NEK2, MAPK and Aurora B were shown to be important (Musacchio and Salmon, 2007). Although BUB1 and BUBR1 could not be identified, both inhibitors had very narrow target profiles. No significant reduction of Aurora A/B, MAPK and NEK2 binding was observed for either drug in this assay, ruling out all three kinases as direct targets of these agents (Figure S4B).

3.5. Elevated Plk1 inhibitor concentrations lead to reduced Aurora B activity

Several lines of evidence propose convincingly that the inhibition of Aurora B blocks chromosome segregation, overrides the SAC and perturbs spindle dynamics in mitosis (Lens et al., 2010; Vader and Lens, 2008). In vertebrate cells, the inhibition of Aurora B abrogates the SAC and, as a
Figure 5 – Dose-dependent effects of Plk1 inhibition on Aurora B localization and the level of mitotic markers in Survivin- or Fox M1-expressing cells. (A) HeLa cells were treated with the indicated concentrations of BI 2536 for 24 h and labeled using DAPI or antibodies for BubR1, Aurora B and α-Tubulin for analysis by immunofluorescence microscopy. Scale bar, 10 μm. (B) Lysates of HeLa cells treated with nocodazole or increasing
consequence, leads to mitotic exit (Ditchfield et al., 2003; Hauf et al., 2003; Kallio et al., 2002). Remarkably, the treatment of Plk1-depleted cells with the small molecule Hesperadin, which inhibits Aurora B kinase activity, caused Cyclin B1 and Securin degradation (Sumara et al., 2004). Thus, the kinase activity of Aurora B is essential for maintaining SAC arrest induced by Plk1 inhibition. Our western blot analyses of different Plk1 inhibitor-treated cell types at high inhibitor concentrations demonstrated reduced levels of phosphorylated Histone H3 at Serine 10 (Figure 1B, Figure 2B, Figures S1B–S3B), which is a substrate of Aurora B, and reduced autophosphorylation of the T-loops within the kinase domains of Aurora B/C, an event that is a prerequisite for efficient kinase activity (Figure 1B, Figures S1B–S3B) indicating the possibility that Aurora B activity is downregulated under these conditions.

To address this issue we performed at first in vitro kinase assays, that confirmed that BI 2536 and BI 6727 are inhibitors of Plk1 with different potencies (Rudolph et al., 2009; Steegmaier et al., 2007), but also demonstrated that neither counteracts the kinase activity of purified Aurora B directly in the range of concentrations tested (0.1–1000 nM) (Figure 3A). We considered the possibility that the in vitro kinase assay using highly purified enzymes does not reflect the physiological activity of Aurora B in living cells. Therefore, we immunoprecipitated Aurora B from cancer cell lines and primary cells treated with BI 2536 and performed kinase assays. We observed a dose-dependent increase of activity up to ~100 nM, reflecting the enrichment of cells in mitosis with high Aurora B activity, and a decline in Aurora B activity at elevated concentrations of BI 2536 (>100–500 nM) (Figure 3B). Plk1 immunoprecipitated from BI 6727-treated cells showed also a biphasic, dose-dependent pattern of activity (Figure 3B). The analysis of BI 6727-treated cells revealed that the decline of Aurora B activity at elevated concentrations was less pronounced compared to BI 2536 (Figure 3B). These results support the hypothesis that deregulation of Aurora B by an unknown mechanism might be contributing to the Plk1 inhibitor-induced silencing of the SAC. We decided to test whether the inhibition of Plk1 per se might contribute to the down-regulation of Aurora B activity.

### 3.6. Exploring the influence of Plk1 inhibition on Cyclin B1 levels and Aurora B activity in cells

To explore the influence of Plk1 on SAC activity, we analyzed human retinal pigment epithelial cells that express wild-type Plk1 (Plk1wt) or an analogue-sensitive Plk1 allele (Plk1as), which shows a profound resistance to BI 2536 (Burkard et al., 2012). When exposed to increasing concentrations of both inhibitors, only Plk1wt could be enriched in G2/M (Figure 4A, B). Levels of Cyclin B1, pH3, Aurora B and Phospho-Aurora B had a peak at 50–100 nM BI 2536 (Figure 4B). The kinase assay of immunoprecipitated Aurora B revealed a peak of Aurora B activity between 100 and 500 nM BI 2536 in Plk1wt cells followed by a decline of activity (Figure 4C) and decreasing levels of Cyclin B1, pH3 and Phospho-Aurora-B as BI 2536 concentrations increased (Figure 4B). The proliferation of cells expressing Plk1wt at concentrations up to ~100 nM was clearly inhibited (Figure 4D). At concentrations >~100–500 nM the anti-proliferative effect came to a halt and was partially reversed (Figure 4D). The analysis of caspase-3/7 activity and annexin staining revealed stagnating or slightly reduced levels of apoptosis at high BI 2536 levels in Plk1wt cells compared to a concentration of 100 nM (Figure 4E).

While low concentrations (~100 nM) did not affect the growth of Plk1as expressing cells, the anti-proliferative effect at BI 2536 concentrations >100 nM was previously attributed to a general toxicity (Burkard et al., 2012) (Figure 4D). Since SAC activation could be achieved in Plk1wt cells by Plk1 inhibition, isogenic cells expressing Plk1as could not be arrested in mitosis (Figure 4B). As an alternative method for SAC activation we treated cells with paclitaxel and explored the role of Plk1 for Cyclin B1 levels and Aurora B activity in Plk1wt and Plk1as cells at increasing concentrations of BI 2536. While high levels of Phospho-Aurora-B (T232)/Aurora C (T198), Aurora B, Cyclin B1, and pH3 persist in BI 2536-resistant Plk1as cells with increasing doses accompanied by a stable mitotic index (Figure 4F, left and right panels), their levels in Plk1wt cells decline at high concentrations accompanied by descending levels of Cyclin B1 and a decreasing mitotic index (Figure 4F, left and right panels). While the microscopic inspection showed decreasing levels of rounded mitotic Plk1wt cells, the percentage of rounded cells remain almost unchanged in Plk1as cells upon treatment with paclitaxel and increasing concentrations of BI 2536. Under the same treatment we could demonstrate almost unchanged SAC activity by using MPM-2 antibodies in Plkwt cells, and descending SAC activity in Plkwt cells. Our data indicate that in cells treated with paclitaxel high Plk1 activity is required to maintain a SAC-induced mitotic arrest.

### 3.7. The localization of Aurora B under conditions of reduced Plk1 activity

Although these observations could suggest that the activities of SAC and Aurora B depend at least partially on Plk1 activity,
the regulatory mechanisms remain elusive. Immunofluorescence analyses confirmed that upon BI 2536 treatment (Lenart et al., 2007), HeLa cells arrest at prometaphase with a high frequency of monopolar spindles, and show BubR1 staining within kinetochore region of the chromosomes indicating SAC activation (Figure 5A). Whereas at low BI 2536 concentrations (100 nM), Aurora B was detected at the chromosome arms and at the kinetochore/centromere region, at high concentrations (2 μM), BI 2536 induced a partial decondensation of chromosomes and provoked a partial displacement of Aurora B that was accompanied by a reduced co-localization with BubR1 at kinetochores (Figure 5A). Since Aurora B, as component of the CPC, exists in a complex with Survivin and INCENP (Bolton et al., 2002), we tested the dose-dependent composition of this complex in a co-immunoprecipitation. Firstly, we observed descending levels of Aurora B with increasing concentrations of BI 2536 (Figure 5B). Secondly, levels of INCENP and Survivin, which were associated with Aurora B were reduced at high drug concentrations (1–2 μM) compared to a concentration of 100 nM, suggesting a partial disintegration of the CPC (Figure 5B).

3.8. Mimicking Plk1-mediated phosphorylation of Survivin and FoxM 1 for maintaining Cyclin B1 levels and Aurora B activity

Specific protein interactions govern the spatially and temporally distinct subcellular localization of Aurora kinases, and also control their expression and activation (Vader and Lens, 2008). Plk1 has been shown to regulate the cellular level and activity of Aurora B by phosphorylating critical mitotic regulators, such as Fox M1, Mklp2 and Survivin (Chu et al., 2011; Fu et al., 2008; Gruneberg et al., 2006). Since the priming phosphorylation of Survivin by Plk1 is critical for Aurora B localization and activity (Chu et al., 2011), we tested whether mimicking Plk1-mediated Survivin phosphorylation could sustain Aurora B activity at increasing concentration of BI 2536. We transfected HeLa cells with wild-type Survivin, a non-phosphorylatable mutant (S20A) or a phosphomimetic mutant (S20D). Only Survivin-S20D induced stable pH3 and Cyclin B1 signals at increasing concentrations of BI 2536 (Figure 5C). Monitoring the autophosphorylation status of Aurora B at high drug concentrations (1–2 μM) compared to a concentration of 100 nM, suggesting a partial disintegration of the CPC (Figure 5B).

3.9. Treatment with high concentrations of Plk1 inhibitors induces mitotic slippage in acute myeloid leukemia (AML) cells

To explore the physiological significance of these findings, we investigated whether Cyclin B1 and Aurora B are also downregulated in pathological situations, such as AML, a disease for which Plk1 inhibition is being tested in clinical trials (Berg et al., 2012). We studied the dose response of primary AML cells (AML1409, 2972) and long-term cultures of primary AML cells (LTC-AML-FPM5,12) by monitoring the expression of mitotic proteins by Western blot (Figure 6A). The response to both inhibitors was dose-dependent. We observed decreased levels of Aurora B, pH3 and Cyclin B1 (Figure 6A) and reduced Aurora B activity (Figure 6B) at high drug concentrations (1–2 μM) suggesting that SAC activity is weakened in primary cancer cells upon treatment with elevated Plk1 inhibitor concentrations.

4. Discussion

The inhibition of Plk1 causes mitotic arrest followed by an induction of apoptosis in many cancer cell types, which makes it an attractive anti-cancer drug target. Our study revealed that the clinical inhibitors BI 2536 and BI 6727 induced a strong mitotic arrest within the entire range of concentrations tested (10–2000 nM) in cancer cell lines and primary, non-transformed cells. The dose responses of the two compounds BI 2536 and BI 6727 differed, most likely due to their differing potencies (Rudolph et al., 2009; Steegmaier et al., 2007). Remarkably, at concentrations >500 nM BI 2536 descending levels of Cyclin B1, Securin, Aurora B, Plk1 and the appearance of polyploid cells were observed. This observation could indicate that high concentrations of the Plk1 inhibitor induce mitotic slippage or failure of cytokinesis. While BI 2536 concentration up to 100 nM reduced the proliferative activity of HeLa cells and other cell types, unexpectedly at increasing concentrations (>100 nM) the anti-proliferative effect came to a halt and was partially reversed with a surviving fraction of up to 10–20%.

Previous studies on the treatment of cancer cells with Taxol revealed that death during mitotic arrest is associated with high Cdk1/Cyclin B1 activity leading to the inactivation of two anti-apoptotic proteins by extensive Mcl-1 phosphorylation/degradation and Bcl-XL phosphorylation (Sakurikar et al., 2012). Low to medium concentrations of BI 2536 (50–500 nM) that induce strong mitotic arrest accompanied by high levels of Cyclin B1, Plk1 and pH3 led to a prominent Mcl-1 degradation and Bcl-XL phosphorylation. At higher BI 2536 concentrations Mcl-1 levels increased and the Bcl-XL phosphorylation decreased suggesting that the anti-apoptotic functions of both proteins is restored, which corroborates our results obtained in three-dimensional colony formation assays showing that at higher concentrations (1 μM) the surviving fractions increased again compared to 10–100 nM BI 2536.

Since a putative mitotic slippage contrasts with the effects expected from studies reporting RNAi-mediated Plk1
depletion in cancer cells (Liu and Erikson, 2003; Spankuch-Schmitt et al., 2002a; Sumara et al., 2004), we decided to analyze the specificity of the small-molecule inhibitors BI 2536 and BI 6727 in more detail. Owing to evolutionary conservation of the ATP-binding site, both Plk1 inhibitors that target this site might promiscuously inhibit multiple kinases. In addition, there is close homology between the kinase domains of Aurora kinases and Plk1. Indeed, the Aurora kinase domain had been used to model the Plk1 kinase domain (Lowery et al., 2005) prior to the determination of the Plk1 crystal structure. Based on the reported inhibition of Plk1 by an Aurora kinase inhibitor with sub-micromolar potency (Kothe et al., 2007), we tested whether Plk1 inhibitors target Aurora B or other SAC kinases directly. We combined the specific nature of small-molecule kinase inhibitors with large-scale quantitative chemical proteomics. In our study, Kinobeads captured approximately two-thirds of the expressed kinome; in combination with quantitative MS, this approach allowed us to study drug binding under physiological conditions. This profiling revealed the high selectivity of BI 2536 and BI 6727, but it did not identify kinases that regulate directly the SAC within the sub-kinome investigated.

Figure 6 — Dose-dependent effects of Plk1 inhibitors in primary AML cells. (A) Lysates of bone marrow cells and long-term AML culture (LTC-AML) were immunoblotted for Plk1, Phospho-Aurora B (T232)/Aurora C (T198), pH3, Cdk1, Cyclin B1 and β-Actin. (B) Following 24 h treatment with BI 2536 or BI 6727, immunoprecipitated Plk1 and Aurora B were subjected to kinase assays using Histone H1/H3 as substrates. For all panels, one representative image of three independent experiments is shown.
Based on this result the question remains whether Plk1 inhibition effects the activity of an important SAC component indirectly. Our comparison of cells expressing a BI 2536-resistant mutant of Plk1 or wild-type Plk1 revealed that full SAC activity requires Plk1 activity in cells, that were treated with paclitaxel for SAC activation: When Plk1 activity falls under a certain threshold at high BI 2536 concentrations, levels of Phospho-Aurora B, Aurora B, Cyclin B1 and pH3 start to decline suggesting that mitotic slippage occurs. The critical role of Plk1 for checkpoint activity was previously suggested by different studies showing that the loss of the Plk1-specific 3F3/2 phosphoepitope is associated with a significant reduction in the levels of kinetochore proteins (Mad2, Cenp-E, Hec1/Ndc80 and Spc24) that play important roles in checkpoint signaling (Ahonen et al., 2005). Moreover, the centromeric activation of Aurora B requires several factors, including substrate-priming phosphorylation by Plk1 and Haspin (Rosasco-Nitcher et al., 2008). Furthermore, Survivin phosphorylation at S20 by Plk1 was shown to control Aurora B activity at the centromere (Chu et al., 2011), whereas Plk1-specific phosphorylation of Mklp2 controls the delocalization of Aurora B from the kinetochores to the central spindle (Santamaria et al., 2007). In our study we used an antibody that recognizes Phospho-Aurora B (T232)/Aurora C (T198) to determine the activation of Aurora kinases. The treatment of different cancer cell lines (HeLa, MDA-MB-468) and primary non-cancer cells with higher doses of both inhibitors demonstrated descending levels of Phospho-Aurora B (T232) and phosphorylated Histone H3 at Serine 10, a direct target of Aurora B. Mimicking the phosphorylation of Fox M1 and Survivin by Plk1 restored at least partially endogenous levels and activity of Aurora B which is crucial for maintaining a level of SAC activity that warrants mitotic arrest.

Rather than targeting Plk1 by using ATP-competitive inhibitors, the PBD, which regulates the protein–protein interaction of Plk1, is ideally suited as an alternative target site. The PBD is unique to the family of Plks and has been shown to be essential for the functions of Plk1. A high-throughput screening assay led to the identification of poloxin (a PBD inhibitor), the first non-peptidic inhibitor that blocks protein–protein interactions of the Plk1 PBD (Reindl et al., 2008a, 2008b). Treatment of cancer cells with poloxin induced mitotic arrest and led to a precise recapitulation of the phenotypical effect of PBD overexpression, whereby the localization of Plk1 was impaired. The antitumor activity of poloxin was demonstrated recently (Yuan et al., 2011). Considering that Plk1 kinase activity is required to maintain SAC activity, the alternative use of PBD-inhibitors like poloxin for the inhibition of Plk1 might be suited to maintain sufficient enzymatic activity of Plk1 to warrant the SAC signaling. However, this functional aspect requires future investigations.

In addition, we observed decreasing activity of Cdk1/Cyclin B1 at high inhibitor concentrations. Considering the key role of Cdk1/Cyclin B1 for SAC activity (Brito and Rieder, 2006), further experiments are required to elucidate whether Cyclin B1 degradation or Aurora B inhibition or a concerted action of both events initiate mitotic slippage at high inhibitor levels. Our recent observations revealed that the concerted action of Cdk1/Cyclin B1 and Plk1 inhibits the activation of caspase-8 (Matthess et al., 2014). Considering this result inhibition of Plk1 might lower also the threshold for the activation of caspases, that are relevant under condition of prolonged mitotic arrest. Moreover, we cannot rule out that Plk1 controls upstream regulators of Aurora B activity other than Fox M1 or Survivin. However, the observed degradation of checkpoint proteins is likely to contribute to the inactivation of the mitotic checkpoint. Although in previous RNAi-based studies, Plk1 levels were substantially reduced (Liu and Erikson, 2003; Spankuch-Schmitt et al., 2002a; Sumara et al., 2004), residual amounts of Plk1 might allow for sufficient checkpoint activity to induce a pronounced M phase arrest. Since BI 2536 and BI 6727 also inhibit Plk2 and Plk3, it will be of interest to study their putative contribution to the regulation of SAC signaling under stressful mitotic conditions. There is evidence showing that Plk2 and Plk3 act as tumor suppressors through their functions in the p53 signaling network, which guards the cell against various stress signals. As long as we do not know whether the inhibition of Plk1 by BI 2536 and BI 6727 is always dominant over the inhibition of Plk2 and Plk3, the contribution of individual member of the Plk family, especially Plk2 and Plk3, remains to be elucidated.

As first reported for treatments that stabilize microtubules (Clute and Pines, 1999), we also found that Cyclin B1 levels progressively decline during a mitotic block induced by Plk1 inhibitors that induce mitotic catastrophe. A slow, but continuous degradation of Cyclin B1 drives cells out of mitosis. We propose a biphasic model: a first phase of SAC activation and mitotic arrest at low Plk1 inhibitor concentrations followed by a second phase characterized by mitotic arrest and mitotic slippage, in which elevated inhibitor concentrations force Plk1 activity below a certain threshold, leading to the reduced SAC activity that is associated with deregulated Aurora B activity and the decline of Cyclin B1 levels.

5. Conclusion

It is encouraging that clinical responses are being observed so early in the development timeline of novel drugs targeting Plk1 (Schoffski, 2009). However, our study demonstrates that elevated concentrations of BI 2536 and BI 6727 above a certain threshold increase mitotic slippage and the likelihood of improved cancer cell survival. The concentrations of BI 6727 used in this study for the treatment of cells in culture are clinically relevant, because similar concentrations were determined in our analyses of bone marrow from AML patients in phase I/II clinical trials with BI 6727 (Bug, 2011). Thus, the determination of a therapeutic window that results in reduced effects on normal tissues while optimizing effects on tumors is necessary to increase the likelihood of beneficial outcomes with these inhibitors.

Conflict of interests

The authors declare no potential conflict of interest.
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Appendix A.
Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.07.020.

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