Polo-like Kinase 1 (Plk1) as a Novel Drug Target in Chronic Myeloid Leukemia: Overriding Imatinib Resistance with the Plk1 Inhibitor BI 2536

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Abstract

In most patients with chronic myeloid leukemia (CML), the disease can be kept under control using the BCR/ABL kinase inhibitor imatinib. Nevertheless, resistance or intolerance to imatinib and other BCR/ABL inhibitors may occur during therapy. Therefore, CML research is focusing on novel targets and targeted drugs. Polo-like kinase 1 (Plk1) is a serine/threonine kinase that plays an essential role in mitosis. In this study, we examined the expression of Plk1 in CML cells and its potential role as a therapeutic target. Plk1 was found to be expressed in phosphorylated form in the CML cell line K562 as well as in primary CML cells in all patients tested. Inhibition of BCR/ABL by imatinib or nilotinib (AMN107) led to decreased expression of the Plk1 protein in CML cells, suggesting that BCR/ABL promotes Plk1 generation. Silencing of Plk1 in CML cells by a small interfering RNA approach was followed by cell cycle arrest and apoptosis. Furthermore, the Plk1-targeting drug BI 2536 was found to inhibit proliferation of imatinib-sensitive and imatinib-resistant CML cells, including leukemic cells, carrying the T315 mutation of BCR/ABL with reasonable IC₅₀ values (1–50 nmol/L). The growth-inhibitory effects of BI 2536 on CML cells were found to be associated with cell cycle arrest and apoptosis. Moreover, BI 2536 was found to synergize with imatinib and nilotinib in producing growth inhibition in CML cells. In conclusion, Plk1 is expressed in CML cells and may represent a novel, interesting target in imatinib-sensitive and imatinib-resistant CML.

Introduction

Chronic myeloid leukemia (CML) is a stem cell disease defined by the chromosomal translocation t(9;22) (1–3). The related oncogene, BCR/ABL, encodes a 210-kDa oncoprotein, BCR/ABL, which exhibits constitutive tyrosine kinase activity (1–3). Various downstream signaling pathways, survival molecules, and cell cycle regulators contribute to BCR/ABL-induced transformation (1–3). These pathways and growth regulators are considered to act together to trigger proliferation and survival in leukemic cells in CML (1–3).

The BCR/ABL kinase inhibitor imatinib is regarded as standard first-line treatment in CML (4–8). Most patients in chronic phase CML can be driven into complete cytogenetic remission with imatinib (4–7). Nevertheless, resistance against imatinib can occur, especially in accelerated phase and blast phase (9–12). In these patients, BCR/ABL point mutations are often detectable (9–12). In other patients, BCR/ABL amplification or additional pro-oncogenic hits are found (9–12). Imatinib-resistant patients often respond to a second-generation BCR/ABL tyrosine kinase inhibitor (TKI) such as nilotinib (AMN107) or dasatinib (13–17). Some of these patients may enter long-lasting complete cytogenetic remission (13–17). However, in many cases, resistance to second-generation TKI develops (15–17). A special problem is the T315I mutation, which confers resistance against most BCR/ABL TKIs (16–19). In other patients, BCR/ABL-independent molecules triggering survival or cell cycle progression may be activated and contribute to resistance. Therefore, new compounds and strategies have been considered to overcome drug resistance in CML (20, 21). One attractive approach may be to combine BCR/ABL TKI with other targeted drugs to achieve synergistic effects (20, 21).

Polo-like kinase 1 (Plk1) is a serine/threonine kinase that plays an essential role in mitosis and cell cycle progression in normal and neoplastic cells (22–24). Plk1 has recently been introduced as a potential therapeutic target in oncology.

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Table 1. Patients’ characteristics and response of leukemic cells to BI 2536

**A. Patients**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (y)</th>
<th>Gender</th>
<th>Phase</th>
<th>BCR/ABL mutations</th>
<th>Previous therapies</th>
<th>Leukocyte count (G/L)</th>
<th>BI 2536 IC50 (nmol/L)</th>
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<tbody>
<tr>
<td>#1</td>
<td>34</td>
<td>M</td>
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<td>Hydroxyurea</td>
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<td>Hydroxyurea</td>
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<td>60</td>
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<td>CP</td>
<td>E255K</td>
<td>Hydroxyurea IFN-alpha Cyclophosphamide BM transplantation Imatinib resistant Nilotinib resistant Dasatinib resistant</td>
<td>41</td>
<td>10–15</td>
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<tr>
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<td>CP</td>
<td>F359V</td>
<td>Hydroxyurea IFN-alpha Cytarabine Imatinib resistant Nilotinib resistant</td>
<td>13</td>
<td>5–10</td>
</tr>
<tr>
<td>#22</td>
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<td>CP</td>
<td>n.d.</td>
<td>Hydroxyurea Imatinib resistant</td>
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<td>10–25</td>
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<td>#23 (1st visit)</td>
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<td>M</td>
<td>CP</td>
<td>L387M</td>
<td>Hydroxyurea Imatinib resistant</td>
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<td>#23 (2nd visit)</td>
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<td>M</td>
<td>CP</td>
<td>G250E E255K T315I</td>
<td>Hydroxyurea Imatinib resistant Dasatinib resistant</td>
<td>12</td>
<td>1–5</td>
</tr>
<tr>
<td>#23 (3rd visit)</td>
<td>43</td>
<td>M</td>
<td>BC</td>
<td>E255K</td>
<td>Hydroxyurea Imatinib resistant Dasatinib resistant BM transplantation</td>
<td>43</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

(Continued on the following page)
Indeed, pharmacologic Plk1 inhibitors counteract cell cycle progression and growth in neoplastic cells (31–33). One of these inhibitors is BI 2536 (32–34). This drug has recently been applied to patients with acute myeloid leukemia (AML) and advanced solid tumors in clinical trials (35, 36).

### Table 1. Patients’ characteristics and response of leukemic cells to BI 2536 (Cont’d)

#### A. Patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (y)</th>
<th>Gender</th>
<th>Phase</th>
<th>BCR/ABL mutations</th>
<th>Previous therapies</th>
<th>Leukocyte count (G/L)</th>
<th>BI 2536 IC₅₀ (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#24 (1st visit)</td>
<td>62</td>
<td>M</td>
<td>CP</td>
<td>T315I</td>
<td>Hydroxyurea Fludarabine BM transplantation</td>
<td>29</td>
<td>25–50</td>
</tr>
<tr>
<td>#24 (2nd visit)</td>
<td>62</td>
<td>M</td>
<td>BC (ly)</td>
<td>T315I</td>
<td>Hydroxyurea Fludarabine BM transplantation</td>
<td>330</td>
<td>25–50</td>
</tr>
<tr>
<td>#25</td>
<td>48</td>
<td>F</td>
<td>AP</td>
<td>T315I</td>
<td>Hydroxyurea Fludarabine BM transplantation</td>
<td>27</td>
<td>25–50</td>
</tr>
</tbody>
</table>

#### B. Cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BI 2536 IC₅₀ (nmol/L)</th>
<th>Combination with imatinib (range/ratio)*</th>
<th>CI for “BI 2536 + imatinib”</th>
<th>Combination with nilotinib (range/ratio)*</th>
<th>CI for “BI 2536 + nilotinib”</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>5–10 nmol/L</td>
<td>BI 2536: 1–6 nmol/L Imatinib: 20–120 nmol/L Ratio: 1:20</td>
<td>0.069–0.732 (synergistic)</td>
<td>BI 2536: 1–6 nmol/L Nilotinib: 2–12 nmol/L Ratio: 1:2</td>
<td>0.001–0.382 (synergistic)</td>
</tr>
<tr>
<td>Imatinib-resistant K562</td>
<td>5–10 nmol/L</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>Native Ba/F3 Ba/F3p210WT</td>
<td>&gt;1 μmol/L</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>Ba/F3p210T315I</td>
<td>10–50 nmol/L</td>
<td>BI 2536: 5–40 nmol/L Imatinib: 0.5–8 μmol/L Ratio: 1:100; 1:200</td>
<td>0.723–1.170 (additive-synergistic)</td>
<td>BI 2536: 5–40 nmol/L Nilotinib: 0.3–1 μmol/L Ratio: 1:20; 1:100</td>
<td>0.176–0.680 (synergistic)</td>
</tr>
<tr>
<td>Ba/F3p210E255K</td>
<td>10–50 nmol/L</td>
<td>BI 2536: 10–20 nmol/L Imatinib: 0.5–1 μmol/L Ratio: 1:50</td>
<td>0.383–0.742 (synergistic)</td>
<td>BI 2536: 10–20 nmol/L Nilotinib: 10–20 nmol/L Ratio: 1:1</td>
<td>0.317–0.520 (synergistic)</td>
</tr>
<tr>
<td>Ba/F3p210M351T</td>
<td>10–50 nmol/L</td>
<td>BI 2536: 10–20 nmol/L Imatinib: 0.5–1 μmol/L Ratio: 1:50</td>
<td>0.197–0.316 (synergistic)</td>
<td>BI 2536: 10–20 nmol/L Nilotinib: 10–20 nmol/L Ratio: 1:1</td>
<td>0.060–0.089 (synergistic)</td>
</tr>
<tr>
<td>Ba/F3p210Y253F</td>
<td>10–50 nmol/L</td>
<td>BI 2536: 10–20 nmol/L Imatinib: 0.5–1 μmol/L Ratio: 1:50</td>
<td>0.341–0.475 (synergistic)</td>
<td>BI 2536: 10–20 nmol/L Nilotinib: 10–20 nmol/L Ratio: 1:1</td>
<td>0.484–0.862 (synergistic)</td>
</tr>
<tr>
<td>Ba/F3p210E396P</td>
<td>10–50 nmol/L</td>
<td>BI 2536: 10–20 nmol/L Imatinib: 0.5–1 μmol/L Ratio: 1:50</td>
<td>0.050–0.068 (synergistic)</td>
<td>BI 2536: 10–20 nmol/L Nilotinib: 10–20 nmol/L Ratio: 1:1</td>
<td>0.157–0.192 (synergistic)</td>
</tr>
</tbody>
</table>

NOTE: Responses of cells to BI 2536 were assessed by [³H]thymidine uptake. CI values were calculated with CalcuSyn software. Abbreviations: M, male; F, female; CP, chronic phase; AP, accelerated phase; BC, blast phase; ly, lymphatic; n.t., not tested; n.d., not detected. *Drug combinations were applied at a fixed ratio of concentrations.
The aims of the present study were to determine whether Plk1 is expressed in activated form in imatinib-naive and imatinib-resistant CML cells and whether Plk1 would represent a potential therapeutic target in CML.

Materials and Methods

Patients and isolation of primary CML cells. Primary CML cells were obtained from the peripheral blood (PB) of 25 patients with CML. The patients’ characteristics are shown in Table 1. For control experiments, bone marrow (BM) cells from 17 lymphoma patients without BM involvement, 1 with cutaneous mastocytosis, 2 with AML in complete remission, and 3 without hematologic disease were used. All samples were collected during routine investigations. The study was approved by the ethics committee (Medical University of Vienna). All patients gave written informed consent before BM or PB was obtained.

Reagents. The Plk1 inhibitor BI 2536 was kindly provided by Dr. Dorothea Rudolph (Boehringer Ingelheim, Vienna, Austria). Imatinib and nilotinib (AMN107) were kindly provided by Drs. Paul Manley and Elisabeth Buchdunger (Novartis Pharma AG, Basel, Switzerland). Stock solutions of drugs were prepared by dissolving in DMSO (Merck). The phosphoinositide 3-kinase (PI3K) inhibitor LY294002, mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitor PD98059, c-Jun NH2-terminal kinase (JNK) inhibitor SP600125, p38 inhibitor SB203580, and mammalian target of rapamycin (mTOR) inhibitor rapamycin were from Calbiochem; bortezomib was from Janssen-Cilag; RPMI 1640, folic acid, heparin, and propidium iodide was from Sigma. The Philadelphia chromosome–positive CML cell line K562 was maintained in RPMI 1640 containing 10% FCS and antibiotics. All samples were collected during routine investigations. The study was approved by the ethics committee (Medical University of Vienna). All patients gave written informed consent before BM or PB was obtained.

Cells and culture conditions. The Philadelphia chromosome–positive CML cell line K562 was maintained in RPMI 1640 and 10% FCS. Imatinib-sensitive and imatinib-resistant K562 cells (37) were used. Imatinib-resistant K562 cells were maintained in imatinib (1 μmol/L). We also examined parental Ba/F3 cells and Ba/F3 cells expressing wild-type BCR/ABL (Ba/F3p210WT) or various BCR/ABL mutants: Ba/F3p210T315I, Ba/F3p210E255K, Ba/F3p210M351T, Ba/F3p210Y253F, and Ba/F3p210T315I, kindly provided by Dr. Michael Deininger (Oregon Health and Science University Cancer Institute, Portland, OR; ref. 38). Primary CML cells were isolated from PB and layered over Ficoll to isolate mononuclear cells. All cells were cultured in RPMI 1640 containing 10% FCS and antibiotics. In a separate set of experiments, Ba/F3 cells were cultured in the presence or absence of interleukin-3 (1 ng/mL).

Detection of Plk1 mRNA by reverse transcription-PCR and quantitative PCR. For reverse transcription-PCR (RT-PCR) analysis and real-time PCR, RNA was isolated using the RNeasy kit (Qiagen). cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and random primers (both from Invitrogen, Inc.) according to the manufacturer’s instructions. Primers used to amplify human Plk1 were: 5′-CCCATCTCTCAGGCTACCGAG-3′ (forward) and 5′-AAGAGCCACCCACCTGTT-3′ (reverse). PCR conditions were as follows: annealing, 30 s (65°C); extension, 1 min (72°C); denaturation, 30 s (94°C); 30 cycles. Equal loading was confirmed by determining β-actin mRNA levels using published primers (39, 40). Real-time PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using iTaq SYBB Green Supermix with ROX (Bio-Rad). Plk1 mRNA levels were normalized against β-actin mRNA levels. Results were expressed as ΔCt values (ΔCt = CPlk1 − Cβ-actin).

Detection of phosphorylated and total Plk1 by immunocytochemistry. Immunocytochemistry was performed on cytosin preparations of primary CML cells, normal BM cells, and K562 cells as described (40). For detection of total Plk1, a polyclonal rabbit anti-Plk1 antibody (work dilution, 1:250; Santa Cruz Biotechnology) and a second-step biotinylated goat anti-rabbit IgG (Biocare) were used. Streptavidin-alkaline-phosphatase complex (Biocare) served as chromogen. Antibody reactivity was made visible by neofuchsin.

Analysis of cell cycle progression, mitosis, and apoptosis. K562 cells were incubated in control medium or in the presence of BI 2536 (1 μmol/L), imatinib (1 μmol/L), nilotinib (1 μmol/L), LY294002 (20 μmol/L), PD98059 (50 μmol/L), SB203580 (10 μmol/L), SP600125 (10 μmol/L), or rapamycin (1 μmol/L) for up to 24 h. In time course experiments, cells were incubated with BI 2536 for 1, 5, 15, and 240 min. Thereafter, immunoprecipitation and Western blotting were performed as described (41) using anti-Plk1 antibody (1:1,000). For immunoperoxidase, lysates from 107 cells were incubated with 5 μL of anti-Plk1 antibody and protein G-Sepharose beads (Amersham). Phosphorylated BCR/ABL (p-BCR/ABL) was detected at 150 to 250 kDa using anti-phosphoprotein antibody 4G10 (Upstate Biotechnology).

Silencing of Plk1 with small interfering RNA. Plk1 small interfering RNA (siRNA; sequence: 5′-GUAACCCUC- CUUAAAUAUdTdT-3′; ref. 29) and control siRNA against luciferase (5′-CUUACGCUGAGAUCUUCAAdTdT-3′) were synthesized by Dharmacon Research. The siRNAs (200 nmol/L) were transfected into K562 cells using Lipofectin (Invitrogen) according to the manufacturer’s instructions. Twenty-four and 48 h after transfection, cells were spun on cytosin slides, and the numbers of mitotic and apoptotic cells were determined by light microscopy. In addition, cells were subjected to cell cycle analysis and Annexin V/propidium iodide staining. Plk1 knockdown was confirmed by Western blotting.
medium for 12, 24, 48, or 96 h. The percentage of mitotic and apoptotic cells was quantified by Wright-Giemsa staining. TUNEL assay was performed by In Situ Cell Death Detection kit, Fluorescein (Roche Diagnostics) as described (39, 40). Flow cytometric analysis of apoptosis was performed using Annexin V-FITC (Alexis Biochemicals; refs. 39, 40). Staining reactions were analyzed by flow cytometry on a FACScan.

**Measurement of cell proliferation.** Cell lines and primary CML cells were incubated with various concentrations of BI 2536 (100 pmol/L to 1 μmol/L). Then, [3H]thymidine uptake was evaluated as described (39, 40). To determine potential additive or synergistic drug effects, BCR/ABL+ cells were exposed to various combinations of BI 2536 and imatinib or BI 2536 and nilotinib at a fixed ratio of drug concentrations. All experiments were performed in triplicates.

**Statistical analysis.** To determine the significance in differences in growth and apoptosis, the Student’s t test for dependent samples was applied. Results were considered significant when P was <0.05. Drug interactions (additive, synergistic, and antagonistic) were determined by calculating combination index (CI) values using CalcuSyn software (BioSoft; ref. 42). A CI value of 1 indicates an additive effect, whereas CI values below 1 indicate synergistic drug effects.

**Results**

**Plk1 is expressed in activated form in CML cells.** As assessed by real-time PCR, Plk1 mRNA levels were found to be higher in primary CML cells (16 patients) compared with normal BM cells [16 donors; ΔC_T BM control (10.1 ± 1.5) versus CML (8.5 ± 1.2); P < 0.05]. Figure 1A shows the expression of Plk1 mRNA in CML cells detected by conventional RT-PCR. Next, we examined Plk1 protein expression in CML cells using antibodies against total Plk1 and p-Plk1. As assessed by immunocytochemistry, K562 cells and primary CML cells were found to express substantial amounts of Plk1 and p-Plk1, whereas normal BM cells expressed only trace amounts of Plk1/p-Plk1 (Fig. 1B). Expression of the Plk1 protein in CML cells (K562) could also be confirmed by Western blotting (Fig. 1C).

**Expression of Plk1 in leukemic cells is BCR/ABL dependent.** To ask whether Plk1 expression is regulated by BCR/ABL, imatinib-sensitive and imatinib-resistant K562 cells were incubated with imatinib and nilotinib for 24 hours. As visible in Fig. 1C, targeting of BCR/ABL was followed by complete downregulation of Plk1 protein expression, suggesting that Plk1 is a BCR/ABL-dependent survival factor. Recent data suggest that p38, a BCR/ABL downstream kinase, directly binds to and activates Plk1 (43). To examine signaling pathways involved in Plk1 expression, K562 cells were incubated in control medium or in LY294002 (20 μmol/L), PD98059 (50 μmol/L), rapamycin (1 μmol/L), SB203580 (10 μmol/L), SP600125 (10 μmol/L), or nilotinib (1 μmol/L) for 24 h before immunoprecipitation and Western blot analysis were performed.

**Figure 1.** Plk1 is expressed in CML cells in a BCR/ABL-dependent manner. A, RT-PCR was performed using cDNA from normal BM (Co BM; n = 3), PB from 16 CML patients, and primers specific for Plk1 and β-actin. B, immunocytochemistry was performed in K562 cells, primary CML cells, and normal BM cells using antibodies against Plk1 (top) or p-Plk1 (bottom). Patients’ numbers (#) refer to Table 1. C, Western blotting and immunoprecipitation were performed with imatinib-sensitive and imatinib-resistant (res.) K562 cells kept in the presence or absence of imatinib or nilotinib (each 1 μmol/L) for 24 h using anti-phosphoprotein antibody 4G10 for detection of p-BCR/ABL and antibodies against Plk1 or β-actin. D, K562 cells were incubated in control medium or in LY294002 (20 μmol/L), PD98059 (50 μmol/L), rapamycin (1 μmol/L), SB203580 (10 μmol/L), SP600125 (10 μmol/L), or nilotinib (1 μmol/L) for 24 h before immunoprecipitation and Western blot analysis were performed.
Targeting of Plk1 with specific siRNA counteracts cell cycle progression and viability of K562 cells. To clarify the functional role of Plk1 in CML cells, a siRNA against Plk1 was applied in K562 cells (Fig. 2A). As visible in Fig. 2B and C, the siRNA-induced Plk1 knockdown was followed by a substantial increase in the percentage of cells arrested in mitosis. As determined by flow cytometry, cells were found to be arrested in the G2-M phase of the cell cycle after treatment with Plk1 siRNA for 24 hours (Fig. 2D). However, later, these cells displayed clear signs of apoptosis, and 48 hours after transfection, the numbers of apoptotic cells increased substantially compared with control, which was demonstrable by light microscopy (Fig. 2B and C) and Annexin V/propidium iodide staining (Fig. 2D). These results suggest that Plk1 is essential for cell cycle progression and survival of CML cells.

BI 2536 counteracts Plk1 expression and induces cell cycle arrest and subsequent apoptosis in K562 cells. In a next step, BI 2536, a pharmacologic Plk1 inhibitor, was applied. K562 cells were incubated in control medium or in 1 μmol/L BI 2536 for 4 or 24 hours. As visible in Fig. 3A, the Plk1 protein was found to be downregulated by BI 2536 within 4 hours. We then applied BI 2536 for 1, 5, or 15 minutes. Again, BI 2536 was found to downregulate expression of Plk1 in K562 cells at all time points (data not shown). To evaluate BI 2536 effects on cell cycle progression and viability, K562 cells were incubated with various concentrations of BI 2536 for 12, 24, 48, and 96 hours. BI 2536 was found to induce mitotic arrest in K562 cells within 12 hours and induced apoptosis after 48 hours. Figure 3B shows the time-dependent effect of BI 2536 (100 nmol/L) on the numbers of mitotic and apoptotic cells. To confirm these results, cell cycle analysis was performed after 24 hours. As visible in Fig. 3C, the percentage of cells in G2-M phase was significantly increased after incubation with 100 nmol/L BI 2536. After 48 hours, the proapoptotic effects of BI 2536 were confirmed by flow cytometry and TUNEL assay (Fig. 3D).

BI 2536 inhibits growth of imatinib-sensitive and imatinib-resistant BCR/ABL-positive cells. We next examined the effects of BI 2536 on proliferation of BCR/ABL-positive cells in [3H]thymidine uptake experiments. As visible in Table 1 and Supplementary Fig. S1, BI 2536 was found to inhibit the growth of all BCR/ABL+ cells tested, with IC50 values...
ranging from 1 to 50 nmol/L. No apparent differences in IC50 were found when comparing imatinib-sensitive CML cells with imatinib-resistant cells, including primary cells and Ba/F3 cells carrying BCR/ABL T315I (Table 1). These data suggest that BI 2536 inhibits the proliferation of CML cells regardless of their response to imatinib or presence of BCR/ABL mutations. No significant effect of BI 2536 was observed in parental Ba/F3 cells, and in normal BM cells, the effect of BI 2536 was less pronounced than in CML cells (IC50 >100 nmol/L; Supplementary Fig. S1).

**BI 2536 effects on K562 cells are reverted by the proteasome inhibitor bortezomib.** The unexpected rapidity with which BI 2536 downregulates the expression of Plk1 in K562 cells prompted us to ask whether protein degradation processes are involved. Once deactivated by drugs, protein kinases may be cleared rapidly from the cytoplasm via proteasomal degradation. To determine whether this may apply to Plk1 downregulation induced by BI 2536, K562 cells were coincubated with 1 μmol/L BI 2536 and 260 nmol/L bortezomib. As shown in Fig. 4A, bortezomib was found to antagonize the effects of BI 2536 on Plk1 protein expression, suggesting that Plk1, after deactivated by BI 2536, is degraded via the proteasome. Furthermore, bortezomib was found to counteract the mitotic arrest produced by BI 2536 (Fig. 4B).

**Figure 3.** BI 2536 regulates Plk1 expression and induces cell cycle arrest and subsequent apoptosis in K562 cells. A, K562 cells were kept in control medium or 1 μmol/L BI 2536 for 4 or 24 h. Thereafter, immunoprecipitation and Western blotting were performed using antibodies against Plk1 or β-actin. B, left, K562 cells were incubated with control medium (top) or 100 nmol/L BI 2536 (bottom) and then stained with Wright-Giemsa; right, K562 cells were incubated for different time periods in BI 2536 (100 nmol/L). The percentage of mitotic cells (gray columns) and apoptotic cells (black columns) was determined by microscopy. Results represent the mean ± SD of three independent experiments. C, K562 cells were kept in control medium (top panel and gray columns in bottom panel) or in 100 nmol/L BI 2536 (middle panel and black columns in bottom panel) for 24 h. Then, cell cycle distribution was analyzed by flow cytometry. The percentage of cells in G0-G1, G2-M, and S phase was calculated. Bottom, results represent the mean ± SD of three independent experiments. D, K562 cells were kept in control medium or in various concentrations of BI 2536 as indicated. After 48 h, cells were harvested and subjected to combined Annexin V/propidium iodide staining (top) or TUNEL assay (bottom).
Figure 4. Effects of drug combinations on growth of CML cells. A and B, K562 cells were cultured in control medium or 1 μmol/L BI 2536 alone or in combination with 260 nmol/L bortezomib for 4 h (A) or 16 h (B). A, immunoprecipitation and Western blotting were performed using antibodies against Plk1 or β-actin (loading control). B, the percentage of mitotic cells was determined by Wright-Giemsa staining. Results represent the mean ± SD of three independent experiments. C, cell lines (top) or primary CML cells (bottom) were incubated with imatinib, nilotinib, or BI 2536 as single drugs (▪-▪, imatinib; ▪-▪, nilotinib; ▪-▪, BI 2536) or in combination (▴-▴) at 37°C for 48 h. Then, [3H]thymidine uptake was measured. Results are expressed as percent of control and represent the mean ± SD of triplicates. Asterisk, CI < 1. Patients’ numbers (#) refer to Table 1.
No cooperative (additive or synergistic) effects of BI 2536 and bortezomib on proliferation or induction of apoptosis in K562 cells were seen (data not shown). Together, these data suggest that protein degradation through the proteasome may be associated with induction of cell death produced by BI 2536.

**BI 2536 synergizes with TKI in countering growth of CML cells.** Drug combinations may represent an important option in drug-resistant CML. To determine whether BI 2536 and TKI synergize in countering proliferation, K562 cells, Ba/F3 cells expressing BCR/ABL mutants, and primary CML cells were incubated with suboptimal concentrations of BI 2536 as single agent or in combination with TKI. As visible in Fig. 4C and Supplementary Fig. S2, BI 2536 was found to synergize with imatinib and nilotinib in inhibiting growth of imatinib-resistant CML cells. Synergistic effects between BI 2536 and TKI were observed with all BCR/ABL mutants tested, including T315I (Fig. 4C; Supplementary Fig. S2).

**Discussion**

In most CML patients, the disease can be kept under control with imatinib (4–7). In case of resistance or intolerance, a second-generation TKI may be prescribed (11–17). However, these patients may develop resistance against all available TKIs, which represents a clinical challenge. Therefore, current research focuses on additional drug targets with the hope to overcome resistance. Plk1 has recently been identified as a potential therapeutic target in various tumors and acute leukemias (24–30). We here show that Plk1 is expressed in CML cells and that the Plk1-targeting drug BI 2536 inhibits growth and survival in leukemic cells. Effects of BI 2536 were seen in imatinib-sensitive and imatinib-resistant CML, including leukemic clones carrying the BCR/ABL mutation T315I that introduces resistance against most available TKIs. These data suggest that Plk1 is a potential new target in CML-cells. The CML cell line K562 was also found to express p-Plk1, confirming the data of Masuda and colleagues (44).

Thus far, little is known about the regulation of expression and activation of Plk1 in neoplastic cells. In our study, the BCR/ABL-targeting TKI imatinib and nilotinib were found to downregulate expression of Plk1 in K562 cells, suggesting that Plk1 expression is BCR/ABL dependent. To define BCR/ABL downstream signal pathways involved in Plk1 expression, we applied signal transduction inhibitors. The PI3K inhibitor LY294002 and the p38 inhibitor SB203580, and to a lesser degree inhibitors of MEK and mTOR, were found to downregulate expression of Plk1 in K562 cells. These data suggest that multiple signaling pathways are involved in the regulation of expression of Plk1.

Recent data suggest that Plk1 is a major regulator of cell cycle progression (24, 25). We examined the function of Plk1 in CML cells by applying Plk1 siRNA. The Plk1 knockdown was followed by cell cycle arrest and subsequent apoptosis in K562 cells, confirming data obtained in solid tumor cells (29, 30). These observations suggest that Plk1 is a regulator of cell growth and thus a potential target in CML cells.

BI 2536 is a pharmacologic inhibitor of Plk1 known to block the growth of various neoplastic cells (32–35). In this study, BI 2536 induced growth inhibition and apoptosis in K562 cells and primary CML cells in all patients tested. In untreated patients, the IC₅₀ values for BI 2536 ranged from 1 to 15 nmol/L, which is within the pharmacologic range of the drug. A similar IC₅₀ value was found in K562 cells, confirming previous data (33). One of the most intriguing effects of BI 2536 was cell cycle arrest observed after a relatively short incubation time. In fact, >50% of all cells treated with BI 2536 showed mitotic arrest after 24 hours, whereas apoptosis occurred at a later time point (48 hours), suggesting that Plk1 is a primary target of BI 2536 in CML cells, whereas apoptosis may be a secondary event (45).

An unexpected observation was that BI 2536 rapidly downregulated expression of total Plk1 in CML cells. Because de-activated target proteins are often degraded rapidly through the proteasome, we applied the proteasome inhibitor bortezomib. This drug was indeed found to counteract the BI 2536–induced downregulation of Plk1 expression in K562 cells, suggesting that the BI 2536–targeted form of Plk1 may be degraded by a proteasome-dependent pathway.

Resistance to imatinib is an emerging problem in the treatment of CML (9–11). We asked whether BI 2536 would exert growth-inhibitory effects in imatinib-resistant CML cells and Ba/F3 cells expressing various imatinib-resistant mutants of BCR/ABL. BI 2536 was found to block proliferation in all cell lines tested, with IC₅₀ values ranging from 5 to 15 nmol/L for K562 cells and from 10 to 50 nmol/L for the Ba/F3 clones analyzed, without major differences observed when comparing IC₅₀ values in the various BCR/ABL mutants examined. Similarly, BI 2536 produced growth arrest in primary CML cells in all imatinib-resistant patients tested, with similar IC₅₀ values, including CML cells harboring BCR/ABL T315I, a mutant conferring resistance against all available TKIs.

Several different treatment concepts in CML focus on drug combinations (39, 40, 46). We observed synergistic effects when combining BI 2536 with imatinib or nilotinib. Synergistic drug actions were seen not only in K562 cells and untreated patients but also in imatinib-resistant cells, including those carrying BCR/ABL T315I. The mechanism of synergy between BI 2536 and BCR/ABL TKI remains unknown. One possibility may be that the cell cycle arrest produced by BI 2536 leads to enhanced responsiveness against imatinib/nilotinib. A similar mechanism has been proposed for imatinib and cisplatin in squamous cell carcinomas (47). Another explanation may be that BI 2536 leads to enhanced drug uptake and/or accumulation of TKI in leukemic cells. A direct effect of BI 2536 on BCR/ABL could be excluded. All in all, our data suggest that drug combinations using BI 2536 and TKI exert substantial antileukemic effects in TKI-resistant CML. However, not all drug combinations may produce such cooperative effects in CML, as exemplified for “imatinib + bortezomib” treatment.

Together, our study shows that Plk1 represents an interesting target in CML. Clinical trials are now warranted to define the value of this new target and to confirm anti-CML effects of these drugs.
Disclosure of Potential Conflicts of Interest

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Polo-like Kinase 1 (Plk1) as a Novel Drug Target in Chronic Myeloid Leukemia: Overriding Imatinib Resistance with the Plk1 Inhibitor BI 2536

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