Polo-Like Kinase 1 is a Therapeutic Target in High-Risk Neuroblastoma

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Introduction

Neuroblastoma is the most common extracranial solid tumor in childhood and accounts for roughly 15% of pediatric oncology deaths (1). The clinical courses of patients with neuroblastoma are highly variable, ranging from spontaneous regression to fatal progression of the disease. Spontaneous tumor involution is regularly observed in infants <18-month old with localized or stage 4S disease, which is defined by the age of the patient (<1 year) and dissemination limited to special sites. On the other hand, approximately half of the patients are currently classified to be at high risk and these children have remained a therapeutic challenge for pediatric oncologists. Although neuroblastoma treatment has been significantly improved over the past decades, the overall survival for high-risk patients has remained less than 40% despite intensive multimodal therapy (1, 2). Thus, novel effective treatment strategies are urgently needed to improve long-term outcome of current high-risk neuroblastoma patients.

A crucial step in tumorigenesis is the loss of cell cycle regulation control (3–5). Protein phosphorylation by specific kinases is an important posttranscriptional process in the modulation of cell cycle kinetics and cell division (6, 7). The serine/threonine-specific Polo-like kinase 1 (PLK1) promotes cell cycle progression by regulating multiple steps during mitosis. PLK1 is a target of the DNA damage checkpoint and is essential for mitotic entry after recovery from DNA-damage induced arrest (8). Elevated PLK1 expression has been found in numerous cancer entities and has been implicated to contribute to tumorigenesis. The oncogenic properties of PLK1 are believed to be due to its role in driving cell cycle progression (9). Accordingly, NIH3T3 overexpressing PLK1 exhibit a transformed phenotype as demonstrated by the ability of these cells to grow in soft agar and to form tumors in nude mice (10). On the other hand, studies comprising a variety of human cancer...
Translational Relevance

High-risk neuroblastoma has remained a therapeutic challenge to pediatric oncologists. Long-term survival of high-risk patients is still below 40% despite intensive multimodal treatment, and new effective treatment regimens are thus urgently needed. Although the understanding of neuroblastoma pathogenesis has been substantially improved over the last years, few potential molecular targets for novel therapeutic strategies have been identified. In this study, we show that expression of the serine-threonine kinase PLK1 is strongly associated with high-risk features in neuroblastoma, and that the PLK1-specific inhibitor BI 2536 significantly decreases neuroblastoma cell viability in vitro and abrogates neuroblastoma tumor growth in xenograft models in vivo at low nanomolar concentrations. Taking the encouraging results of phase I and II clinical trials on BI 2536 in adult solid tumors into account, these data strongly suggest that targeting PLK1 by small molecule inhibitors could represent a new promising therapeutic strategy in the treatment of high-risk neuroblastoma patients.

cell lines have shown that depletion of PLK1 induces growth inhibition and induction of apoptosis in cancer cells, but not in normal human cells (11, 12). Identification of aberrant mitotic kinase signaling pathways provides novel opportunities for cancer-drug discovery. Recently, a potent small-molecule inhibitor, the dihydropyridinidone derivative BI 2536, has been described to selectively inhibit mammalian PLK1 at low nanomolar concentrations in an ATP-competitive manner (8, 13, 14). In 32 human cancer cell lines tested, BI 2536 inhibited proliferation independently of tissue origin and oncogenic status, with half-maximal inhibitory concentration (IC50) values in the range of 2–25 nmol/L. Furthermore, regression of large tumors at well-tolerated intravenous dose regimens was observed in various xenograft models of human cancer (15). In addition, BI 2536 was well tolerated in phase I and II clinical trials, showing a favorable pharmacokinetic profile and antitumor activity in patients with various advanced solid tumors (8, 16, 17). In this study, we aimed at evaluating PLK1 as a potential therapeutic target in high-risk neuroblastoma. For this purpose, we determined PLK1 expression levels in a large cohort of primary neuroblastomas and investigated the effect of PLK1 inhibition by BI 2536 on neuroblastoma growth in vitro and in vivo.

Materials and Methods

Gene expression analysis and patient characteristics

Gene expression profiles were generated from 476 primary neuroblastoma samples (stage 1, n = 118; stage 2, n = 78; stage 3, n = 71; stage 4, n = 148; stage 4S, n = 61) using a 44 K oligonucleotide microarray as described elsewhere (18). MYCN-amplification was observed in 67 tumors, while it was absent in 405 tumors (not determined, n = 4). Patients’ age at diagnosis ranged from 0 to 296 months (median age, 13 months). Median follow-up for patients without fatal events was 5.6 years (range, 0.4–18.1 years). Stage was classified according to the International Neuroblastoma Staging System (19). Patient characteristics and treatment data as well as expression profiling data are available within the in-house MIAME compliant database iCHIP (http://www.icnip.de). This includes raw as well as processed data. Comprehensive and actual patient information is associated with the related experiments, standard operating procedures and protocols for treatment procedures are included according to the MIAME standard. In addition, all data are also available through ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae; Accession: E-MTAB-179).

Western blot analysis

Endogenous PLK1 protein levels were analyzed in neuroblastoma cell lines CHP-212, IMR-32, Kelly, SH-EP, SH-SYSY, SK-N-AS and SK-N-BE(2) and 8 primary tumor samples. The NuPAGE electrophoresis system (Novex Mini Cell, Invitrogen) and semi-dry blotting system (Biometra) were used for protein analysis. For each sample, 10–50 μg of total protein were separated by SDS-PAGE in 4% to 12% bis-Tris gels and transferred to nitrocellulose membranes by semi-dry blotting. The membranes were blocked with 5% dry milk powder in 0.05% Tween 20/phosphate-buffered saline before incubation with primary antibody (monoclonal mouse anti-plk1 WH10005347M1; Sigma-Aldrich Co.) and horseradish peroxidase-labeled secondary goat antiantibody (P0447; Dako). The antigen-antibody complex was detected with Visualizer Spray & Glow (Upstate).

Compound

BI 2536, 4-[(R)-8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5, 6, 7, 8-tetrahydro-pteridin-2-ylamino]-3-methoxy-N-{1-methyl-piperidin-4-yl}-benzamide, was provided by Boehringer Ingelheim GmbH, Germany. For in vitro studies, BI 2536 was dissolved in DMSO at a concentration of 10 mmol/L and stored at −80°C. For in vivo studies, BI 2536 was dissolved in DMSO at a concentration of 5 mg/mL and stored at −80°C.

Cell culture

The neuroblastoma cell lines CHP-212, Kelly, SH-EP, SH-SYSY, SK-N-AS, and SK-N-BE(2) were obtained from the American Tissue Culture Collection (ATCC) and the neuroblastoma cell line IMR-32 was purchased from the DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig). All cell lines were maintained in RPMI-1640 [PAA Laboratories GmbH] supplemented with 10% fetal calf serum (PAA). All cell lines were maintained in a humidified incubator at 37°C with 5% CO2 and passaged at 90% confluence using Accutase (PAA).
**PLK1 is a Therapeutic Target in Neuroblastoma**

**In vitro growth property assays**

Cell viability after BI 2536 exposure was determined in manual cell counting assays. Solutions of BI 2536 (Boehringer Ingelheim Pharma GmbH & Co. KG) were prepared in RPMI-1640 supplemented with 10% FCS containing 0.1% DMSO. CHP-212, IMR-32, Kelly, SH-EP, SH-SY5Y, SK-N-AS, and SK-N-BE(2) were treated with various concentrations of BI 2536 (1 nmol/L, 5 nmol/L, 25 nmol/L, and 125 nmol/L) and the number of viable cells was determined by trypan blue exclusion using a hemocytometer (C-Chip DHC-NO1; Digital Bio Technology Co.). Each measurement was done in triplicate wells 24 hours, 72 hours, and 120 hours after exposure to BI 2536.

The effect of BI 2536 on the cell-cycle profile of neuroblastoma cells was assessed by flow cytometry. SK-N-AS and Kelly were exposed to various concentrations of BI 2536 (1 nmol/L, 5 nmol/L, 25 nmol/L, and 125 nmol/L) for 14 hours and 72 hours and the cell cycle profiles were determined by flow cytometry as described previously (20). Briefly, the cells were harvested with versene, treated with a citric acid buffer (2.1% citric acid and 0.5% Tween 20 in dH2O), and stained using a phosphate buffer (pH 8.0, 7.2 g Na2HPO4 × 2H2O in 100 mL dH2O) containing DAPI. DNA-histograms were obtained by flow cytometry (PAS II, Partec) and the Multicycle program (Phoenix Flow Systems) was used for histogram analysis. Each measurement was done in triplicate.

**BI 2536 treatment of xenograft tumors in nude mice**

Neuroblastoma cells were cultured to 80% confluence, harvested, and suspended in Matrigel (BD Bioscience). Treatment of SK-N-AS and IMR-32 xenografts was studied by weekly subcutaneous inoculation in BALB/c nu/nu line and treatment modality (BI 2536 and control). Nine to ten mice were investigated per cell line and treatment modality (BI 2536 and control). Tumors were assessed using a digital caliper and mean tumor volumes were determined by flow cytometry as described previously (20). DMSO-containing saline was injected into control animals. Six to 10 mice were investigated per cell line and treatment modality (BI 2536 and control). Tumors were assessed by manual cell counting assays. Solutions of BI 2536 (Boehringer Ingelheim Pharma GmbH & Co. KG) were prepared in RPMI-1640 supplemented with 10% FCS containing 0.1% DMSO. CHP-212, IMR-32, Kelly, SH-EP, SH-SY5Y, SK-N-AS, and SK-N-BE(2) were treated with various concentrations of BI 2536 (1 nmol/L, 5 nmol/L, 25 nmol/L, and 125 nmol/L) and the number of viable cells was determined by trypan blue exclusion using a hemocytometer (C-Chip DHC-NO1; Digital Bio Technology Co.). Each measurement was done in triplicate wells 24 hours, 72 hours, and 120 hours after exposure to BI 2536.

**Data analysis and statistics**

Statistical analyses were performed using SPSS software version 17.0 for Microsoft Windows (SPSS Inc.). Log-intensity values of PLK1 mRNA determined by microarray analyses were compared in patient groups defined by MYCN status (normal vs. amplified), age (<18 months vs. >18 months), tumor stage (stage 1–3 and 4S vs. stage 4), and gene expression-based classification (favorable vs. unfavorable) according to our prognostic PAM classifier (21). Two-tailed nonparametric tests (Mann–Whitney U and Kruskal–Wallis test) were used where appropriate to test whether PLK1 expression levels differed statistically in these groups. Kaplan–Meier estimates for event-free survival (EFS) and overall survival (OS) were calculated and compared by log-rank test. Recurrence, progression, and death from disease were considered as events. For multivariate analysis, Cox proportional hazards regression models based on EFS and OS were applied. Cases with missing values were excluded from analysis. The covariates were fitted into a stepwise backward selection. The likelihood ratio test for inclusion was <0.05 and for exclusion was >0.10. In vitro IC50 and IC90 values were calculated from BI 2536 dose–response curves using XLfit 5 software (IDBS Ltd., Guildford, Surrey, UK). Tumor volumes in xenograft model mice were compared using a 2-sided t-test, and survival times were compared using Kaplan–Meier estimates and log-rank test. Statistical significance refers to the average values at the day the experiment was terminated.

**Results**

**High PLK1 transcript levels are associated with unfavorable prognostic markers and poor outcome in neuroblastoma**

To assess whether PLK1 might serve as a potential therapeutic target in high-risk neuroblastoma, we evaluated PLK1 transcript levels in a large cohort of 476 neuroblastoma samples reflecting the whole spectrum of the disease by microarray analysis (18). Similar ranges of PLK1 transcript levels were observed for stage 1–3 tumors and stage 4S tumors, whereas these subgroups exhibited lower expression levels in comparison to stage 4 neuroblastomas (stage 1–3 and 4S vs. stage 4, P < 0.001; Fig. 1A). PLK1 transcript levels were elevated in patients older than 18 months at diagnosis (P < 0.001; Fig. 1B) as well as in tumors with MYCN amplification (P < 0.001; Fig. 1C). Furthermore, high PLK1 expression was significantly associated with unfavorable gene–expression based classification (P < 0.001; Fig. 1D) according to a highly accurate classifier that we have previously defined using the prediction analysis for microarrays algorithm (PAM, Refs. 21 and 22). This predictive signature comprises 144 genes, but does not include PLK1, suggesting that elevated PLK1 mRNA levels are associated with an aggressive molecular neuroblastoma phenotype.

In addition, we determined the association of PLK1 expression levels with patient outcome. For this purpose,
patients were divided into 4 quartiles (n = 119 each) according to the PLK1 expression levels, and Kaplan–Meier estimates were compared by log-rank test. Consistent with the correlation of high PLK1 transcript levels with unfavorable prognostic markers, it turned out that PLK1 expression was gradually associated with poor EFS and OS (Fig. 1E and F). The effect of PLK1 expression on patient outcome was most evident in patients with high expression levels (>75th percentile), who had a significantly worse EFS and OS than those with intermediate-high, intermediate-low, and low PLK1 expression levels (5-year EFS 38.6±4.7% vs. 64.1±4.5% vs. 75.8±4.1% vs. 83.5±3.6%, respectively, and 5-year OS 52.5±5.0% vs. 78.2±4.1% vs. 89.7±3.1% vs. 94.6±2.1%, respectively; both P < 0.001, Fig. 1E and F). Finally, the prognostic value of PLK1 expression was assessed in multivariate Cox regression models based on EFS and OS considering prognostic markers that are currently used for neuroblastoma risk stratification in Germany (Table 1). In these analyses, PLK1 expression turned out to be a significant independent prognostic marker for both EFS and OS (EFS, hazard ratio, 3.798, 95% confidence interval, 1.901–7.585, P < 0.001; OS, hazard ratio, 2.734, 95% confidence interval, 1.068–7.002, P = 0.035).
Together, the strong association of elevated PLK1 transcript levels with unfavorable prognostic markers and poor outcome of neuroblastoma patients suggest that PLK1 could be utilized as a molecular target in high-risk neuroblastoma treatment.

<table>
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<th>Marker</th>
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<th>P</th>
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<td>Model based on EFS</td>
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<td>PLK1 expression (continuous)</td>
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<td>B.</td>
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<td>Model based on OS</td>
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<tr>
<td>PLK1 expression (continuous)</td>
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<td>2.734</td>
<td>1.068–7.002</td>
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<td>1.680–6.133</td>
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*CI, confidence interval; N/S, not significant.

PLK1 protein levels were examined in 8 primary neuroblastoma samples and in 7 cell lines by Western blot hybridization. In primary tumors, the protein level of PLK1 correlated well with PLK1 mRNA expression levels (Fig. 2A).

**Figure 2.** PLK1 protein levels (Western blot analysis) and PLK1 transcript levels (given as log intensities as determined by microarray analysis) in 8 primary A, neuroblastoma samples and B, 7 neuroblastoma cell lines.
Figure 3. Effects of BI 2536 on neuroblastoma cell survival. Cell lines were treated in triplicate with various concentrations of BI 2536 or RPMI-1640 supplemented with 10% FCS containing 0.1% DMSO (control) and counted at the indicated time points (A). Dots, average results of 3 independent experiments; bars, SD. Half-maximal growth inhibition (IC50 values; [nmol/L]) following BI 2536 treatment of neuroblastoma cell lines (B). Changes in cell viability were quantified by trypan blue exclusion and manual cell counting 72 hours after initiation of treatment with BI 2536 or RPMI-1640 supplemented with 10% FCS containing 0.1% DMSO (control). The MYCN status of each cell line is given. SC, single copy; AM, amplified.
miRNA-126, miRNA-125a, and miRNA-499 are downregulated. miRNA-196, miRNA-21, miRNA-141, and miRNA-221 are upregulated. Together, these findings indicate a critical inhibitory effect of BI 2536 on human high-risk neuroblastoma cell growth in general.

BI 2536 abrogates neuroblastoma tumor growth in nu/nu mice

To investigate the effect of BI 2536 treatment on neuroblastoma tumorigenicity in vivo, we examined the ability of BI 2536 to inhibit the growth of subcutaneous IMR-32 and SK-N-AS xenografts in immunodeficient nu/nu mice in 2 different settings.

In the first setting, treatment started 2 or 3 days post tumor cell inoculation, and consecutive cycles of 50 mg/kg BI 2536 were given intravenously once per week. In both IMR-32 and SK-N-AS xenograft models, BI 2536-treated mice revealed almost complete response and showed no significant increase in tumor volume as compared to the primary implant (Fig. 5A). In contrast, all control animals formed tumors that exceeded 1,000 mm³ and had to be sacrificed between days 19 and 37 after inoculation of the tumor cells (Fig. 5A). The final tumor volumes of the treatment groups constituted 1.3% and 9.2% of the tumor volumes of the control groups for IMR-32 and SK-N-AS xenografts, respectively.

In the second setting, treatment of neuroblastoma xenografts was initiated after the tumors had reached a mean volume of 191 ± 61 mm³ (182 ± 52 mm³ in the control group and 201 ± 69 mm³ in the treatment group). Mice were treated with 50 mg/kg body weight BI 2536 intravenously twice per week (15). As shown in Figure 5B, tumor growth was abrogated by this regimen in both xenograft models. At the day the first mouse had to be sacrificed, tumor volumes of the treatment groups accounted for 28.5% and 12.5% of the tumor volumes of the control groups for IMR-32 and SK-N-AS xenografts, respectively. All control animals formed tumors that exceeded 1,000 mm³ and had to be sacrificed between days 8 and 22 after start of the treatment (Fig. 5B). Together, these results demonstrate that low nanomolar concentrations of BI 2536 significantly inhibit neuroblastoma cell growth in vivo.

Discussion

Treatment of high-risk neuroblastoma patients has remained a challenge for pediatric oncologists. Current therapeutic strategies comprise dose-intensive induction chemotherapy, surgery, radiotherapy as well as myeloablative therapy followed by autologous stem cell rescue. In addition, immunotherapy using monoclonal antibodies and differentiation therapy using retinoic acid has been shown to contribute to maintenance of remission (27). Nevertheless, long-term outcome of these children has remained less than 40% (1, 2). In addition, toxicity from current therapy regimens is already significant, giving little room to further intensify conventional chemotherapy. Thus, alternative treatment strategies are urgently needed in order to improve survival and cure rates of these patients.

Targeting key components of the mitotic machinery such as the PLK1 represents a promising strategy in cancer therapy in general (11, 12, 14). Elevat
small-cell lung cancer, melanoma, and hepatoblastoma (9, 28). Moreover, PLK1 expression levels have been observed to correlate with the metastatic potential of tumors (29) and with the prognosis of cancer patients (28, 30–32). Depletion or specific inhibition of PLK1 results in reduced survival of various types of cancer cells in vitro and inhibition of tumor growth in vivo in xenograft models (15, 33, 34). PLK1 inhibition specifically induces spindle assembly checkpoint-induced prometaphase arrest (13), which may avoid some of the severe side effects commonly observed with other antimitotic agents such as taxanes and Vinca alkaloid derivatives that affect many critical cellular processes (e.g., axonal transport) unrelated to mitosis (14, 35).

Currently, several PLK inhibitors from various drug classes are explored in a number of early clinical trials (17). In this study, we demonstrate that elevated PLK1 expression is associated with the presence of unfavorable prognostic markers and poor survival in neuroblastoma. These findings are in line with a previous microarray analysis reporting on an upregulation of PLK1 among other genes involved in cell cycle regulation and chromosome segregation processes in stage 4 and MYCN-amplified neuroblastomas as compared to lower stages without MYCN amplification (36). In our study, we corroborate this result and furthermore show by analysis of a large neuroblastoma cohort representing the whole spectrum of the disease that PLK1 expression is an independent marker of poor outcome in this malignancy. However, in light of the high prognostic accuracy of current risk-stratification strategies and complex DNA- or RNA-based prognostic classifiers (21, 22, 37–39), it remains questionable, whether PLK1 expression will be utilized for risk estimation of neuroblastoma patients in the future. Western blot analysis revealed that PLK1 transcript levels correlate well with PLK1 protein levels (Fig. 2A), which may indicate that measurement of PLK1 mRNA is predictive of its functional activity in neuroblastoma. In addition, the finding of a markedly decreased survival probability in patients with
Figure 5. Antineuroblastoma effect of BI 2536 on IMR-32- and SK-N-AS-tumor growth in immunodeficient nu/nu mice. Tumor volume (left panel) and Kaplan–Meier survival curves (right panel) in 2 different settings: Starting 2–3 days post injection of IMR-32 or SK-N-AS cells into the flank of nude mice, animals received vehicle (IMR-32, n = 9; SK-N-AS, n = 10) or BI 2536 (IMR-32, n = 10; SK-N-AS, n = 10) at doses of 50 mg/kg once weekly via intravenous injection (A). Starting after IMR-32 or SK-N-AS tumors had reached mean volumes of 182 ± 52 mm³ in the control and 201 ± 69 mm³ in the treatment group, animals received vehicle (IMR-32, n = 10; SK-N-AS, n = 6) or BI 2536 (IMR-32, n = 9; SK-N-AS, n = 6) at doses of 50 mg/kg twice weekly via intravenous injection (B). Treatment was continued for the duration of the study. Dots, average volume of 6–10 tumors; bars, 95% confidence interval.
high PLK1 expression in comparison to those with lower expression (>75th percentile vs. <75th percentile. Fig. 1E and F) supports the previously discussed assumption that above some critical level, PLK1 activity may contribute to solid tumor progression in general (31, 40).

Despite major improvements in our understanding of the pathogenetic determinants of neuroblastoma progression over the past decades, only few potential molecular targets for novel therapeutic strategies have been identified. Small molecule inhibitors targeting protein kinases are increasingly attracting attention as promising anticancer drugs in general. However, clinical trials on such inhibitory compounds for high-risk neuroblastoma therapy are still rare. Currently, clinical trials have been initiated investigating the nonselective kinase inhibitors Gefitinib (NCT00135135) and CEP-701 (NCT00084422) as well as the ALK inhibitor PF-02341066 (NCT00939770) in neuroblastoma patients (www.cancer.gov临床 trials). The present study provides a rationale for the potential clinical utility of PLK1 inhibitors in neuroblastoma treatment by demonstrating encouraging antitumor effects of BI 2536 on neuroblastoma cells in vitro and in vivo. We show that inhibition of PLK1 by BI 2536 results in a rapid decrease of viable cells and significantly reduced proliferation. IC50 values were in the low nanomolar range (Fig. 3B), and IC90 values were in the range of therapeutic plasma concentrations achieved after intravenous application of 200 mg BI 2536 in clinical phase I/II trials (8, 41). Treatment with 25 nmol/L BI 2536 induced G2/M phase arrest followed by subsequent tumor cell death (Fig. 4), which is consistent with findings obtained in cell lines of other cancer entities (15, 26). In addition, significant suppression of tumor formation and of established tumor growth was observed in xenograft models treated with BI 2536 (Fig. 5). Collectively, these consistent findings in several neuroblastoma cell lines indicate a strong inhibitory effect of BI 2536 on neuroblastoma cell growth in general.

In conclusion, this study demonstrates that PLK1 expression is elevated in aggressive neuroblastoma, and that inhibition of PLK1 activity by BI 2536 significantly decreases neuroblastoma cell viability in vitro and impedes tumor growth in xenograft models. These findings indicate that PLK1 may play a critical role in tumor progression and may represent a suitable molecular target for inhibitory therapeutic strategies of high-risk neuroblastoma patients. In a limited number of phase I and II clinical trials, treatment with BI 2536 has recently been reported to be well tolerated and to show disease stabilization in 30% to 40% of adult cancer patients with advanced solid tumors (8, 16, 41–43). The current development of novel PLK1 inhibitors with a more favorable pharmacokinetic profile is expected to further improve antitumor activity of PLK1 inhibition in cancer patients (17, 44). Taken together, these data strongly suggest that targeting PLK1 with BI 2536 or its derivatives, either as a single agent or in combination with chemotherapy or radiotherapy, should be pursued as a novel strategy in the treatment of high-risk neuroblastoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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