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

Sandra Ackermann, Felix Goeser, Johannes H. Schulte, Alexander Schramm, Volker Ehemann, Barbara Hero, Angelika Eggert, Frank Berthold, and Matthias Fischer

Abstract

Purpose: High-risk neuroblastoma remains a therapeutic challenge for pediatric oncologists. The Polo-like kinase 1 (PLK1) is highly expressed in many human cancers and is a target of the novel small-molecule inhibitor BI 2536, which has shown promising anticancer activity in adult malignancies. Here, we investigated the effect of BI 2536 on neuroblastoma cells in vitro and in vivo to explore PLK1 as a potential target in high-risk neuroblastoma therapy.

Experimental Design: PLK1 transcript levels were analyzed by microarrays in 476 primary neuroblastoma specimens, and correlation with prognostic markers and patient outcome was examined. To explore the effect of PLK1 inhibition on neuroblastoma cells, 7 cell lines were treated with BI 2536 and changes in growth properties were determined. Furthermore, nude mice with IMR-32 and SK-N-AS xenografts were treated with BI 2536.

Results: PLK1 is highly expressed in unfavorable neuroblastoma and in neuroblastoma cell lines. Expression of PLK1 is associated with unfavorable prognostic markers such as stage 4, age >18 months, MYCN amplification, unfavorable gene expression-based classification, and adverse patient outcome (P < 0.001 each). On treatment with nanomolar doses of BI 2536, all neuroblastoma cell lines analyzed showed significantly reduced proliferation, cell cycle arrest, and cell death. Moreover, BI 2536 abrogated growth of neuroblastoma xenografts in nude mice.

Conclusions: Elevated PLK1 expression is significantly associated with high-risk neuroblastoma and unfavorable patient outcome. Inhibition of PLK1 using BI 2536 exhibits strong antitumor activity on human neuroblastoma cells in vitro and in vivo, opening encouraging new perspectives for the treatment of high-risk neuroblastoma. Clin Cancer Res; 17(4); 1–11. ©2010 AACR.
In this study, we aimed at evaluating PLK1 as a potential molecular target. BI 2536 was dissolved in DMSO at a concentration of 5 mg/mL and stored at −80°C. In vitro studies, BI 2536 was well tolerated in phase I and II clinical trials, showing a pharmacokinetic profile and antitumor activity in inhibiting 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.

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.ichip.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-ae; Accession: E-MTAB-179).
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 in 2 settings: (i) Eight-week-old female athymic NCR (nu/nu) mice were inoculated subcutaneously in the flank with 1.5 × 107 IMR-32 or 1.0 × 107 SK-N-AS neuroblastoma cells in 200-μl Matrigel. Treatment started at day 2 (SK-N-AS) or day 3 (IMR-32) post tumor cell injection. Mice were treated by tail vein injection of 50 mg/kg body weight BI 2536 in 300 μl sodium chloride 0.9% once weekly. DMSO-containing saline was injected into control animals. Nine to ten mice were investigated per cell line and treatment modality (BI 2536 and control). (ii) Eight-week-old female athymic NCR (nu/nu) mice were inoculated subcutaneously in the flank with 1.0 × 107 IMR-32 or SK-N-AS neuroblastoma cells in 200-μl Matrigel. Treatment started when the tumors had reached mean volumes of 182 ± 52 mm3 in the control group and 201 ± 69 mm3 in the treatment group. Mice were treated by tail vein injection of 50 mg/kg body weight BI 2536 in 300 μl sodium chloride 0.9% twice weekly as described previously (15). 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 using a digital caliper and tumor volume was calculated according to the formula (width × length × depth)/2. Mice were sacrificed when tumor volume exceeded 1,000 mm3.

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 P 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 mRNA 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 1.** Multivariate Cox regression models based on EFS and OS considering PLK1 expression (continuous), stage (4 vs. 1, 2, 3, 4S), MYCN status (amplified vs. normal), 1p status (altered vs. normal), and age (>18 months vs. <18 months)

<table>
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<th>Marker</th>
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<th>Hazard ratio</th>
<th>95% CI</th>
<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>N/S</td>
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<tr>
<td></td>
<td>Age (&gt;18 months vs. &lt;18 months)</td>
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<tr>
<td>B.</td>
<td>Model based on OS</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>PLK1 expression (continuous)</td>
<td>2.734</td>
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<td>1.680–6.133</td>
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</tbody>
</table>

*CI, confidence interval; N/S, not significant.

**BI 2536 inhibits proliferation of neuroblastoma cells**

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.](image-url)
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.
In addition, PLK1 protein levels of neuroblastoma cell lines were found to be in the range of protein levels in patients with high PLK1 expression (Fig. 2B). Thus, these tumor cell lines are likely to be suitable models for evaluating the efficacy of PLK1 inhibition in primary tumors.

To examine whether the PLK1 inhibitor BI 2536 affects proliferation of human neuroblastoma cell lines, the neuroblastoma cell lines CHP-212, IMR-32, Kelly, SH-EP, SH-SY5Y, SK-N-AS, and SK-N-BE(2) cells were incubated with 4 different 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 after 24 hours, 72 hours, and 120 hours. Treatment with 25 nmol/L BI 2536 for 72 hours significantly decreased cell viability as compared to DMSO controls in all cell lines analyzed (Fig. 3A). The effect on cell viability was not only independent of the presence of MYCN amplification of the cells (Fig. 3B), but also of biological characteristics such as metastatic or primary tumor tissue derivation (23), morphology (24), or doubling time (25).

We observed IC50 values of 9.85 ± 9.61 nmol/L (Fig. 3B) and IC50 values of 42.05 ± 32.84 nmol/L, which is in the range of previously reported values for cell lines of other tumor entities (15, 26).

Next, we analyzed the effect of BI 2536 exposure on the cell cycle in Kelly and SK-N-AS cells. The cells were treated with 4 different concentrations of BI 2536 (1 nmol/L, 5 nmol/L, 25 nmol/L, and 125 nmol/L) for 14 hours, and the DNA content of the cells was then determined by flow cytometry. On treatment with 25 nmol/L or 125 nmol/L BI 2536 for 14 hours, both cell lines showed a G2/M phase arrest with a 4N DNA content (Fig. 4, upper panels). Prolonged exposure to BI 2536 for 72 hours led to an increase of a characteristic hypodiploid (sub-G1) DNA peak, indicating the accumulation of extensive cell debris due to DNA breakdown and/or apoptosis (Fig. 4, lower panels). 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 injection, 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). Elevated PLK1 levels have been found in many cancer entities as compared to their normal counterparts including breast, colorectal and non...

**Figure 3.** (Continued)
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 4. DNA profiles of exponentially growing Kelly and SK-N-AS cells treated with BI 2536 concentrations ranging from 1 nmol/L to 125 nmol/L for 14 hours (upper panel) and for 72 hours (lower panel). Cells accumulate in G2/M phase (tetraploid DNA content) upon treatment with 25 and 125 nmol/L at 14 hours prior to cell death indicated by an increase of sub-G1 peak at 72 hours.
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 inhibitor Gefitinib (NCT00135135) and CEP-701 (NCT00084422) as well as the ALK inhibitor PF-02341066 (NCT00939770) in neuroblastoma patients (www.cancer.gov/clinicaltrials). 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. IC_{50} values were in the low nanomolar range (Fig. 3B), and IC_{90} 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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References

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