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Small Molecule Kinase Inhibitor Screen Identifies Polo-Like Kinase 1 as a Target for Neuroblastoma Tumor-Initiating Cells

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Abstract

Neuroblastoma (NB) is an often fatal pediatric tumor of neural crest origin. We previously isolated NB tumor-initiating cells (NB TIC) from bone marrow metastases that resemble cancer stem cells and form metastatic NB in immunodeficient animals with as few as ten cells. To identify signaling pathways important for the survival and self-renewal of NB TICs and potential therapeutic targets, we screened a small molecule library of 143 protein kinase inhibitors, including 33 in clinical trials. Cytostatic or cytotoxic drugs were identified that targeted PI3K (phosphoinositide 3-kinase)/Akt, PKC (protein kinase C), Aurora, ErbB2, Trk, and Polo-like kinase 1 (PLK1). Treatment with PLK1 siRNA or low nanomolar concentrations of BI 2536 or BI 6727, PLK1 inhibitors in clinical trials for adult malignancies, were cytotoxic to TICs whereas only micromolar concentrations of the inhibitors were cytotoxic for normal pediatric neural stem cells. Furthermore, BI 2536 significantly inhibited TIC tumor growth in a therapeutic xenograft model, both as a single agent and in combination with irinotecan, an active agent for relapsed NB. Our findings identify candidate kinases that regulate TIC growth and survival and suggest that PLK1 inhibitors are an attractive candidate therapy for metastatic NB. *Cancer Res; 71(4); 1385–95. ©2011 AACR.*

Introduction

Neuroblastoma (NB) is the most common and deadly extracranial solid tumor in children (1, 2). It is an embryonal malignancy thought to arise from the primitive sympathetic neural precursors that normally differentiate to form the sympathetic nervous system. Up to 60% of patients present with widely metastatic disease at diagnosis, typically characterized by bone and bone marrow metastases. Despite intensive treatment regimens, comprising surgery, chemotherapy, and irradiation, high-risk NB patients with relapse in the bone marrow have a long-term survival rate of less than 10% (1, 2). In addition, patients who respond to chemotherapy are at risk for developing long-term complications such as hearing loss, cardiac dysfunction, and infertility. Thus, a search for novel, more potent, and less toxic treatments of NB is warranted.

A growing body of evidence supports the notion that "cancer stem cells" or "tumor-initiating cells" (TIC) are present in multiple malignancies and are responsible for sustaining tumor growth, progression, relapse, and metastases (3–5). Therefore, targeting TICs, particularly from patients with relapsed NB, is an attractive and novel therapeutic approach. We recently identified TICs from NB bone marrow metastases that have several properties of cancer stem cells, including the expression of stem cell markers, the ability to self-renew, and the capability to form metastatic NB in immunodeficient animals with as few as 10 cells (6). These cells, propagated as spheres in serum-free neural stem cell media, are ideal for identifying NB TIC–selective signaling pathways and potential NB therapeutic agents because of their greatly enhanced tumorigenic potential as compared with adherent cell lines established in serum-containing media (6, 7). However, little is known about the signaling pathways required for NB TIC survival and self-renewal from the metastatic bone marrow niche.

To identify NB TIC pathways and novel therapeutic targets for clinical use, we performed a drug screen on bone marrow–derived TICs with a unique collection of pharmacologic kinase inhibitors. We show that Polo-like kinase 1 (PLK1) is required for NB TIC survival *in vitro*. Furthermore, BI 2536, a PLK1 inhibitor currently in clinical trials for adult cancers, significantly inhibits tumor growth in a xenograft model, both as a single agent and in combination with the NB drug irinotecan and is therefore a promising candidate for NB treatment.

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Materials and Methods

Cell culture

NB TICs (NB12, NB88R2, and NB122R) and skin-derived precursor cells (SKP; FS90, FS105, FS107, FS274, FS276, FS280, and FS298) isolated from postnatal foreskin were cultured as previously described (6–9). SMS-KCNR, obtained from Carol Thiele (NIH), and SK-N-AS cells from American Type Culture Collection were cultured as spheres in serum-free neural stem cell media.

Kinase library

A kinase inhibitor library consisting of 143 drugs active against at least 48 individual kinases as primary targets was compiled by the Medicinal Chemistry Platform at the Ontario Institute for Cancer Research (OICR; Supplementary Table 1). The compounds were purchased from a number of vendors: Alexis, Axon Medchem BV, Cayman Chemical, Chemietek, LC Laboratories, Sigma Aldrich and Tocris Bioscience. BI 2536 for *in vitro* and *in vivo* follow-up studies was purchased from Selleck Chemicals. BI 6727 was synthesized according to the procedures provided in the patent literature (WO2007030361).

Primary screening assay

Screening was performed at the SMART Facility of the Samuel Lunenfeld Research Institute, as previously described (10). TICs were dissociated into single cells and seeded at 3,000 cells per well in 100 μL medium in 96-well microplates. Compounds were dissolved in DMSO, re-aliquoted in daughter plates as 1 and 0.2 mmol/L solutions, and added using a pin tool to achieve final concentrations of 1 or 0.2 μmol/L. Drug effects were compared with cells optimally proliferating in 0.1% DMSO alone, whereas wells filled with media served as the background. alamarBlue (10 μL) was added after 72 hours, and fluorescence intensity was measured after 24 hours on a PHERAstar microplate reader, equipped with a λ_{540} excitation/λ_{590} emission filter.

Assay quality, hits selection, and validation

The dimensionless parameters Z'- and Z-factors were used to assess robustness and consistency of the assay. Hits were defined as compounds that caused a signal decrease of at least 45% as compared with controls. Confirmatory tests were performed using 8-point, 3-fold serial dilutions of compounds. Cells were seeded, treated with serially diluted compounds, and tested for alamarBlue reduction as previously.

Sphere formation assay

NB TICs and SKPs from several patients were seeded in triplicate in untreated 96-well microplates at a density of 3,000 cells per well in 50 μL per well, containing 30% SKP conditioned media (10). Compounds were diluted in medium (1:1,000) and immediately added to the cells in a volume of 50 μL (final concentration of DMSO = 0.05%). Cells were retreated 72 hours postplating with drugs and fixed after 6 days with 4% paraformaldehyde (Electron Microscopy Sciences). Sphere number was determined by manual counting, and the results were expressed as the mean sphere number of treated wells as compared with DMSO-treated wells × 100. EC_{50} curves were generated using GraphPad Prism 5 software (GraphPad Software, Inc.).

RNA interference

TICs (NB88R2) were transfected with 500 nmol/L of either scrambled siRNA or a PLK1 siRNA pool (Dharmacon Accell; ThermoFisher) by nucleofection (Microporator). Transfection conditions were as follows: voltage, 1,350V; pulse width, 20 mm; pulse number, 2. Following transfection, cells were seeded in triplicate wells at a density of 1×10^5 per well in 24-well dishes (1 mL medium per well). On specific time points (i.e., 24, 48, and 72 hours), spheres were collected, dissociated, stained with trypan blue, and visually inspected to assess viability by the trypan blue exclusion method.

Western blot analysis

For cell lysis, cells were collected and washed in cold PBS and lysates were prepared in NP-40 lysis buffer. Equal amounts of protein were resolved on 10% polyacrylamide gels and subjected to immunoblotting with the following antibodies: mouse anti-PLK1 (1:1,000; Invitrogen), rabbit anti-cleaved PARP (1:1,000; Cell Signaling), mouse anti-cyclin B1 (1:1;000; Cell Signaling), rabbit anti-p21 (1:1;000; Cell Signaling), rabbit anti-ERK1 (1:10,000; Santa Cruz), and mouse anti-GAPDH (1:10,000; Ambion). Horseradish peroxidase–conjugated goat anti-mouse IgG (1:5,000) and goat anti-rabbit IgG (1:10,000) were used as the secondary antibodies.

Viable cell counts following treatment with BI 2536

A total of 1×10^5 dissociated NB TICs (NB88R2) were seeded in triplicate in 24-well non–tissue culture-treated plates in 1 mL of medium. BI 2536 (10–100 nmol/L) or DMSO was immediately added to the cells. At specific time points, spheres were collected, dissociated, and subjected to a viable cell count by trypan blue exclusion.

Annexin V assay

The ability of BI 2536 to induce apoptosis in NB TICs was determined with an Annexin V-FITC detection kit, used according to the manufacturer's instructions (BD Pharmingen). Briefly, 1×10^6 NB TICs (NB88R2) were plated in 12-well dishes and cultured with various concentrations of BI 2536 (1–100 nmol/L). At various time points (16–40 hours), cells were harvested, stained for Annexin V/propidium iodide (PI), and analyzed on an LSR II flow cytometer. Relative numbers of Annexin V–positive, PI-negative cells were obtained for each time point.

Determination of cellular DNA content

NB TICs (1×10^6) were plated in 12-well dishes and cultured in the presence of either DMSO or BI 2536 (10 and 100 nmol/L). After 16 hours, cells were harvested, washed in PBS, and fixed in 70% ethanol. Fixed cells were treated with RNAse A, stained with PI at 37°C for 1 hour, and analyzed on an LSR II flow cytometer to determine cellular DNA content.

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In vitro PLK1 kinase assay

The assay was performed as previously described (11). Briefly, NB TICs (NB88R2) were dissociated into single cells and treated in the presence of 0.1% DMSO or 100 nmol/L BI 2536 for 3 hours. Cells were then solubilized in NP-40 lysis buffer, and lysates normalized for protein content were incubated with PLK1 monoclonal antibody (Millipore). Immune complexes were recovered with protein A-Sepharose beads and incubated with 5 μCi [γ-^{32}P]ATP at 30°C for 20 minutes and 10 μg of dephosphorylated α-casein (Sigma) in 30 μL of PLK1 kinase buffer (20 mmol/L HEPES, pH 7.4, 50 mmol/L KCl, 10 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L EGTA, 1 μmol/L ATP). The reactions were stopped by adding 20 μL of Laemmli buffer. Samples were separated by SDS-PAGE, and gels were visualized by autoradiography.

Xenograft models

TICs (NB88R2; 3×10^5) were resuspended in PBS, mixed 1:3 with Matrigel (Trevigen), and injected in 30-μL volume into the dermis of 4- to 5-week-old NOD/SCID (nonobese diabetic/severe combined immunodeficient) mice. Drug treatment began when tumor size reached approximately 50 to 100 mm^3. Mice were injected intravenously on 2 consecutive days each week with either BI 2536 (12.5 or 25 mg/kg) or vehicle (0.1N HCl per saline) for a total of 2 to 3 cycles. In the combination study, mice were treated with vehicle (0.1N HCl per saline), 12.5 mg/kg of BI 2536, and/or 10 mg/kg of irinotecan as described in the figure legend.

Assessment of bone marrow toxicity in a xenograft model following treatment with BI 2536

TICs (NB88R2; 3×10^5) were resuspended in PBS, mixed 1:3 with Matrigel (Trevigen), and injected in 30-μL volume into the dermis of 4- to 5-week-old NOD/SCID mice. Drug treatment began when tumor size reached approximately 50 to 100 mm^3. Mice were injected intravenously on 2 consecutive days each week with either BI 2536 ($n = 10$; 25 mg/kg) or vehicle ($n = 10$; 0.1N HCl per saline) for a total of 2 cycles. Blood samples were collected 24 hours after the last drug dose and analyzed via Hemavet Hematology Analyzer (950FS) to access the differences in red blood cell/white blood cell counts.

Statistical analysis

Statistical analyses were performed with Microsoft Excel, using an unpaired, 2-tailed Student's *t* test, with $P < 0.05$ as the significance cutoff. One-way ANOVA was used to determine statistically significant differences from the mean in the combination study *in vivo*.

Results

Small molecule kinase inhibitor screen identifies compounds that target NB TICs

To identify signaling pathways required for NB TIC survival and proliferation, we screened low-passage TIC lines from bone marrow metastases from 3 high-risk NB patients (NB12, NB88R2, and NB122R) with a collection of 143 kinase inhibitors (Fig. 1A). Positive hits were defined as compounds exhibiting more than 45% growth inhibition at 1 μmol/L in at least 1 NB TIC line. To validate the data from the primary screen, we retested the hit compounds using 8-point serial dilutions for each inhibitor and determined from these data EC_{50} values (Fig. 1B). More than 85% of the primary hits were confirmed (23/27), showing excellent assay quality. Interestingly, all of the hit compounds were found to target all 3 NB TIC lines, albeit with different potencies (Fig. 1B). Furthermore, 15 confirmed compounds inhibited NB TIC growth with nanomolar potency (Fig. 1B, marked in green; $EC_{50} < 1$ μmol/L). The identified hit compounds were grouped into clusters on the basis of their reported primary kinase target. For several targets, including phosphoinositide 3-kinase (PI3K)/Akt, protein kinase C (PKC), PLK1, Aurora kinase, ErbB2, and Trk, at least 2 compounds per target were found to exhibit cytotoxicity toward NB TICs (Fig. 1B).

Secondary screen identifies PLK1 as a promising NB target

To further define the most potent and selective compounds, a secondary screen was conducted in which NB TICs were tested in parallel with normal human pediatric SKPs, which are neural crest-like stem cells (8, 9). Similarly to NB TICs, SKPs self-renew *in vitro*, express neural crest progenitor markers such as nestin, and can be differentiated into neural crest lineages but are nontumorigenic. We thus used SKPs as an accessible nontransformed counterpart of NB TICs, suitable for testing potential toxicity of various drugs (10).

A total of 15 hit compounds with nanomolar potency were chosen for secondary screening. As summarized in Table 1, 9 of 15 compounds showed a modest selectivity profile (<10-fold difference in EC_{50} values). However, 6 compounds showed considerable selectivity, resulting in 10-fold to greater than 1,000-fold difference in EC_{50} values (Table 1; highlighted in bold). Of the 6 compounds, PLK1 inhibitors were chosen for further analysis, as PLK1 inhibitors, GW843682X and BI 2536, showed excellent potency as well as more than 10-fold selectivity toward NB TICs than for SKPs (Fig. 2A and B). Both PLK1 inhibitors suppressed NB TIC self-renewal in the nanomolar range ($EC_{50} = 470$ and 6.7 nmol/L, respectively), whereas SKP self-renewal capacity was affected only at micromolar concentrations ($EC_{50} =$ 3.6 and 5.2 μmol/L, respectively), indicating a favorable selectivity profile and a wide therapeutic window (Table 1 and Fig. 2A and B). BI 2536, however, exhibited only micromolar cytotoxicity toward SK-N-AS ($EC_{50} = 3$ μM; data not shown), a bone marrow–derived NB cell line that was established in adherent and serum-containing conditions and that we adapted to spheroid and serum-free culture. BI 2536, an ATP-competitive inhibitor that shows 10,000-fold or greater selectivity for PLK1 relative to 63 other kinases and is currently in phase II clinical trials for adult malignancies (12, 13), was of particular interest because of its specificity for PLK1 and as a potential drug that could be used for NB. A third PLK1 inhibitor, BI 6727, which exhibits an improved

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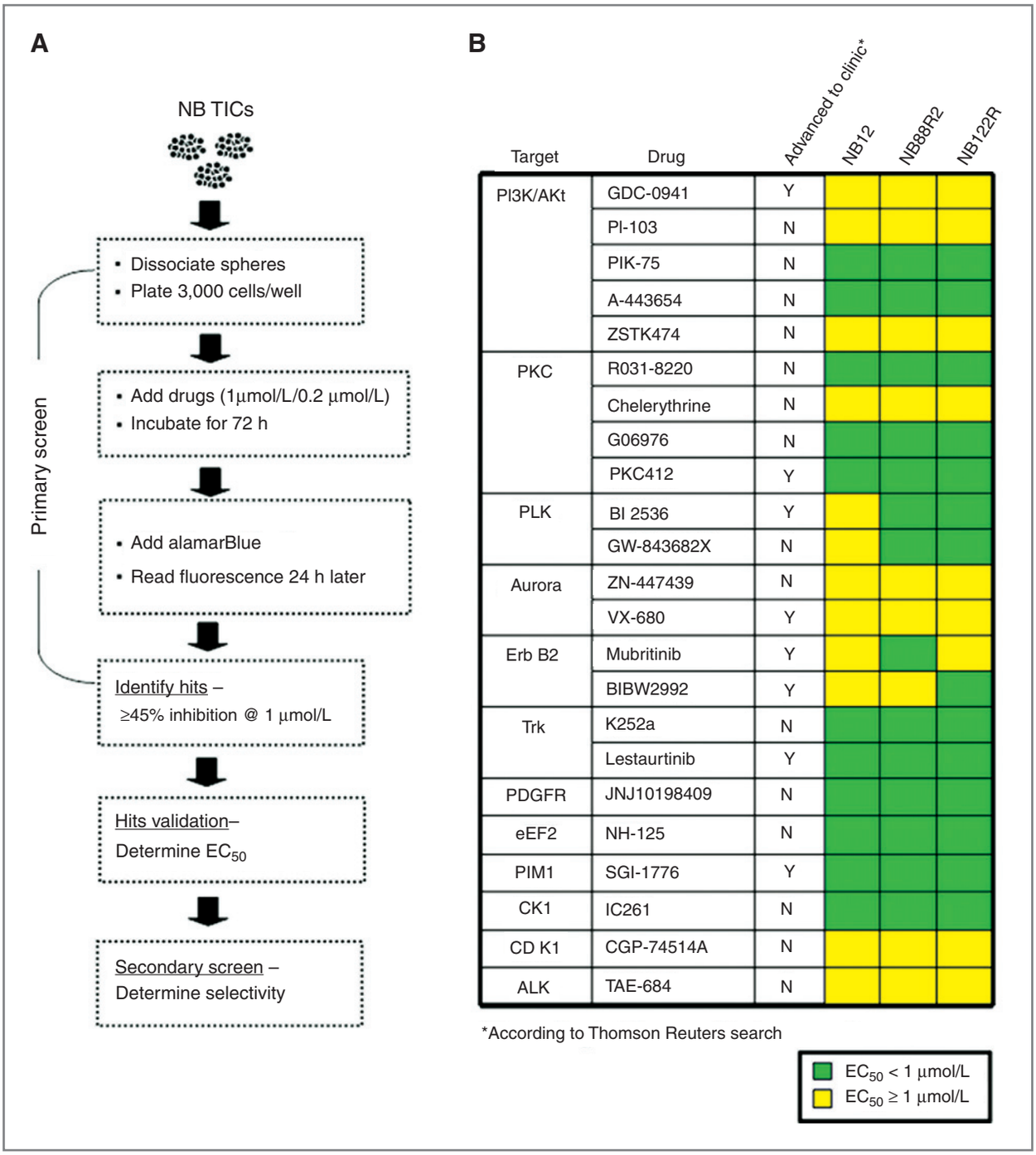

Figure 1. A, detailed design of the screening procedure. B. validation of the data from the primary screen. Compounds that exhibit $EC_{50} < 1$ μmol/L are shown in green, whereas drugs that exhibit $EC_{50} > 1$ μmol/L are shown in yellow.

pharmacokinetic profile as compared with BI 2536 and is currently undergoing evaluation in phase II clinical trials for adult malignancies, was also assessed (14). Similarly to BI 2536, BI 6727 showed nanomolar activity on NB TICs, with an EC_{50} of 21 nmol/L, and an excellent selectivity profile, with an EC_{50} of 2.8 μmol/L on SKPs (Fig. 2A and B).

Suppression of PLK1 levels dramatically decreases TIC survival

To gain further insight into the role of PLK1 in NB TICs, we first assessed PLK1 protein expression via Western blot analysis (Fig. 2C). PLK1 was expressed by a panel of NB TIC lines from relapsed bone marrow metastases and human NB cell

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Table 1. Summary of the secondary screening is presented, which evaluates NB TICs and SKPs sphere formation capacity following treatment with 15 most potent compounds

Target	Drug	EC_{50}, nmol/L		Fold difference
		TIC	SKPs	
PI3K/Akt	A-443654	48.4	207.3	4.3
	PIK-75	58	30	0.51
PKC	R031-8220	590.5	1,370	2.3
	G06976	4,051	9,930	2.5
	PKC412	143.5	102	0.7
PLK	BI 2536	6.7	3,575	533.6
	GW-843682X	470	5,175	11
ErbB2	Mubritinib	579	6,000	10.3
	BIBW2992	2,025	3,945	2
Trk	K252a	691	2,150	3.1
	Lestaurtinib	37.8	1,500	39.7
PDGFR	JNJ10198409	885	>1,000.000	>1.000
eEF2	NH125	202.6	255	1.25
PIM1	SGI-1776	2,300	2,700	1.17
CK1	IC261	329	30,700	93

NOTE: For each compound, EC_{50} is calculated for both NB TICs and SKPs from testing multiple lines at least 2 to 3 times. Compounds that are in shaded cells are the most selective drugs, which show at least 10-fold selectivity between SKPs EC_{50} and TIC EC_{50}.

lines established in serum-containing media conditions and was virtually absent in SKPs (Fig. 2C). This observation is consistent with the notion that PLK1 is selectively overexpressed in tumor cells (15) and provides an explanation for the enhanced sensitivity of NB cells as compared with nontransformed SKPs.

We next confirmed the requirement of PLK1 for NB TIC survival by a transient siRNA knockdown approach. Suppression of PLK1 expression resulted in a 7- to 10-fold reduction in viable cell numbers as compared with cells treated with scrambled siRNA (Fig. 2D). The efficacy of the PLK1 siRNA was confirmed by immunoblotting with anti-PLK1 (Fig. 2D). These results indicate that PLK1 is required for NB TIC survival.

BI 2536 treatment induces cell-cycle arrest and aberrant accumulation of cyclin B1 and p21

We next asked whether BI 2536 targets proteins or pathways in NB TICs that are known to be modulated by PLK1. We hypothesized that BI 2536 acts through cell-cycle disruption, as has been previously reported in various tumor cell lines (11, 13). Following incubation with BI 2536, the percentage of NB TICs in the G_2/M phase of the cell cycle significantly increased, in addition to the emergence of a peak, indicative of cells with 8*n* DNA content (Fig. 3A). Cyclin B1, which is required for cell-cycle progression and is a known downstream PLK1 substrate (11, 16), aberrantly accumulated following incubation with BI 2536 (Fig. 3B and C), indicating that this drug acts in NB TICs in a manner similar to that in other tumor cells. Furthermore, the cyclin-dependent kinase inhibitor p21, a newly identified substrate of PLK1, (16, 17), also accumulated in BI 2536–treated NB TICs (Fig. 3C). Finally, PLK1 kinase activity was inhibited following treatment of NB TICs with BI 2536, as demonstrated by anti-PLK1 immunoprecipitiation followed by *in vitro* PLK1 kinase assay (Fig. 3D). Taken together, these results suggest that cyclin B1 and p21 are targets of PLK1 in NB TICs and that BI 2536 inhibits PLK1 activity and PLK1 downstream effector proteins.

BI 2536 treatment induces cell death via apoptosis

We next asked whether BI 2536, like PLK1 siRNA, induces apoptosis in NB TICs. To assess this, NB TICs were treated with varying concentrations of BI 2536 and viable cells were counted at 24, 48, and 72 hours posttreatment (Fig. 4A). We observed a significant difference in viable cell numbers in BI 2536–treated samples, as compared with DMSO (vehicle)-treated cells, at 24 hours posttreatment. The difference became more pronounced with time, such that at 72 hours there were only approximately 20% viable cells following treatment with 10 nmol/L BI 2536 and approximately 2% to 5% viable cells following treatment with 30 and 100 nmol/L as compared with DMSO-treated cells.

To determine whether the cell death following BI 2536 treatment was due to apoptosis, NB TICs were lysed at different time points posttreatment and the presence of cleaved PARP, a marker associated with apoptosis, was examined. At 24 hours, BI 2536–treated cells gradually accumulated cleaved PARP, in contrast to DMSO-treated cells, suggesting induction of apoptosis (Fig. 4B). To confirm the induction of apoptosis by BI 2536, we evaluated Annexin V expression on the surface of drug-treated cells by flow cytometry. Representative data are shown in

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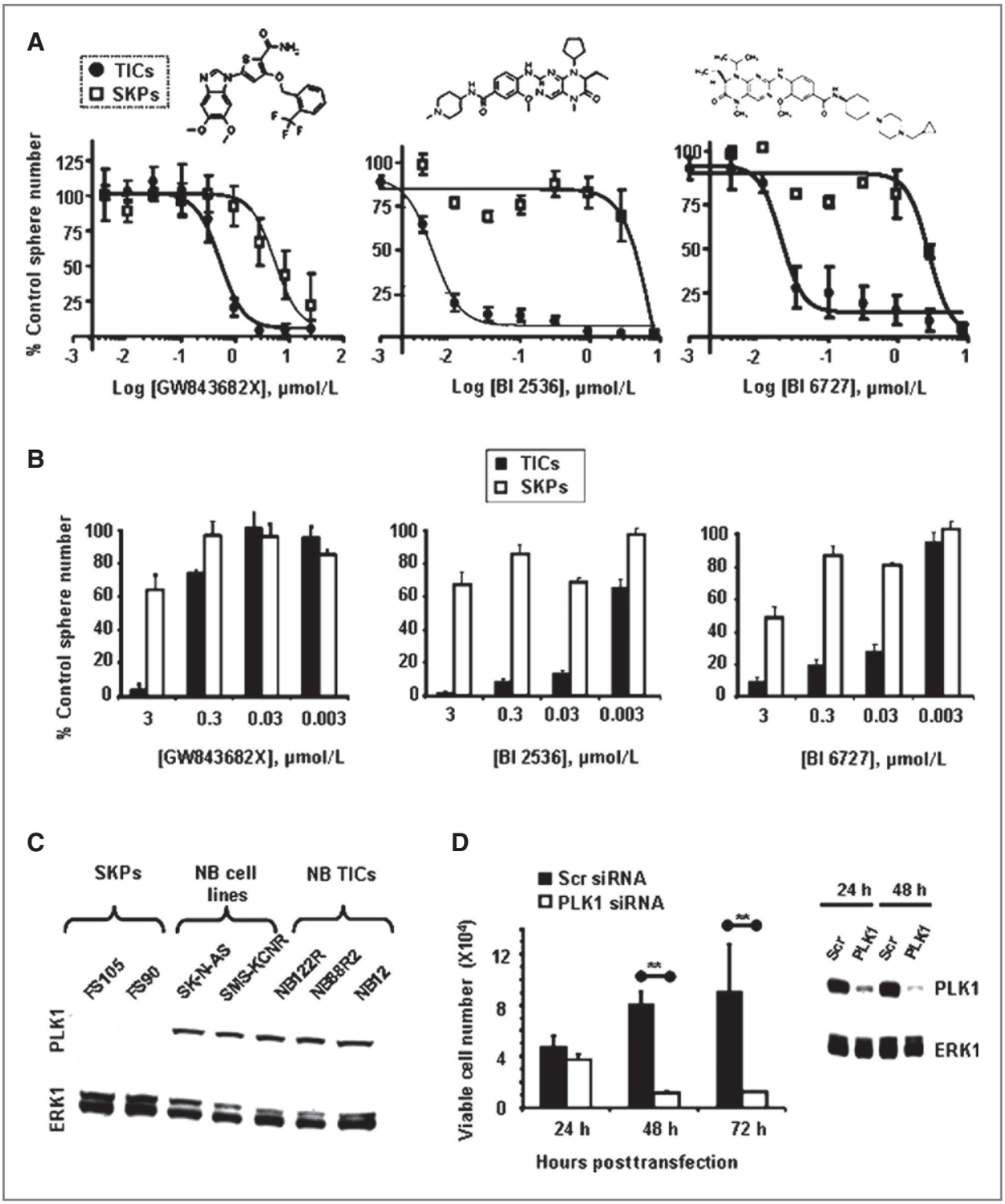

Figure 2. PLK1 disruption is crucial for NB TIC survival and self-renewal. A, EC_{50} curves for 3 PLK1 inhibitors obtained by sphere counting as a read-out. B, quantification of sphere assay data for multiple TIC and SKP lines following treatment with varying concentrations of the 3 compounds ($n = 3$–5). C, PLK1 expression in TICs, NB established cell lines, and SKPs by immunoblotting with anti-PLK1. D, PLK1 siRNA-mediated knockdown in NB88R2 results in decreased PLK1 levels and impaired survival as assessed by cell counting at 24 to 72 hours. $n = 3$; **, $P < 0.01$.

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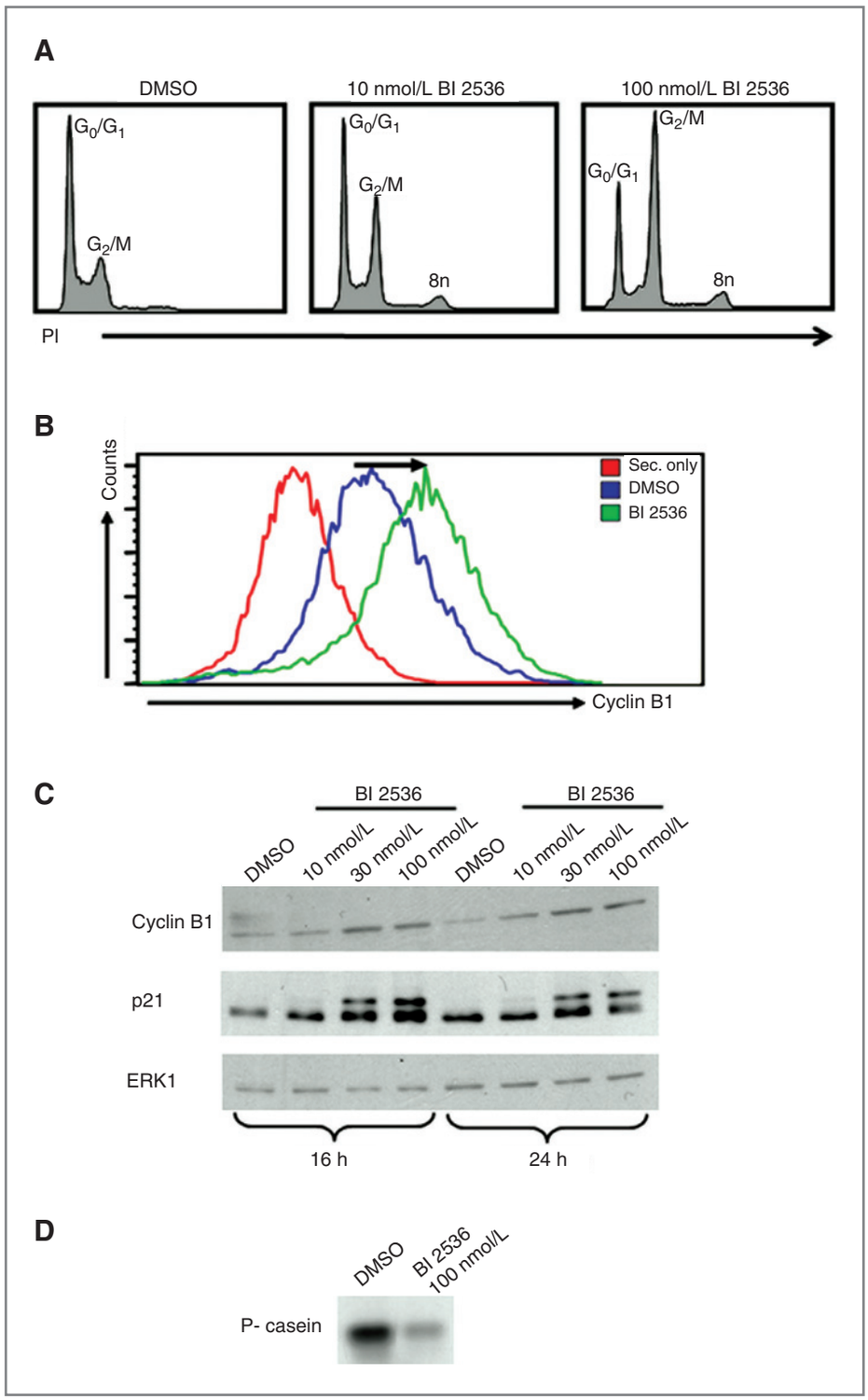

Figure 3. Treatment with BI 2536 induces cell-cycle arrest in NB TICs and aberrant accumulation of cyclin B1 and p21. A, representative histograms for the effect of BI 2536 (10 and 100 nmol/L) on cellular DNA content in NB88R2 ($n = 3$). B, expression of cyclin B1 was assessed in NB88R2 following 24-hour treatment with 100 nmol/L BI 2536. The change in MFI was visualized on LSR II flow cytometer. C, expression of cyclin B1 and p21 was assessed in NB88R2 following 16 and 24-hour treatment with different doses of BI 2536 (10, 30, and 100 nmol/L). ERK1 (extracellular signal regulated kinase 1) was used as the loading control. D, inhibition of PLK1 was assessed by *in vitro* kinase assay following treatment of NB88R2 for 3 hours with BI 2536.

Figure 4C. Annexin V–positive, PI-negative cells, indicative of early apoptotic cells, were present at low levels in cells treated with DMSO or 1 nmol/L BI 2536. This cell population, however, became more prominent at 16 hours posttreatment with 10 or 100 nmol/L BI 2536, reached a peak at 30 hours, and persisted until 40 hours. The quantification of the data is presented in Figure 4D. Taken together, the data indicate that low nanomolar concentrations of BI 2536 induce cell-cycle arrest and cell death by apoptosis.

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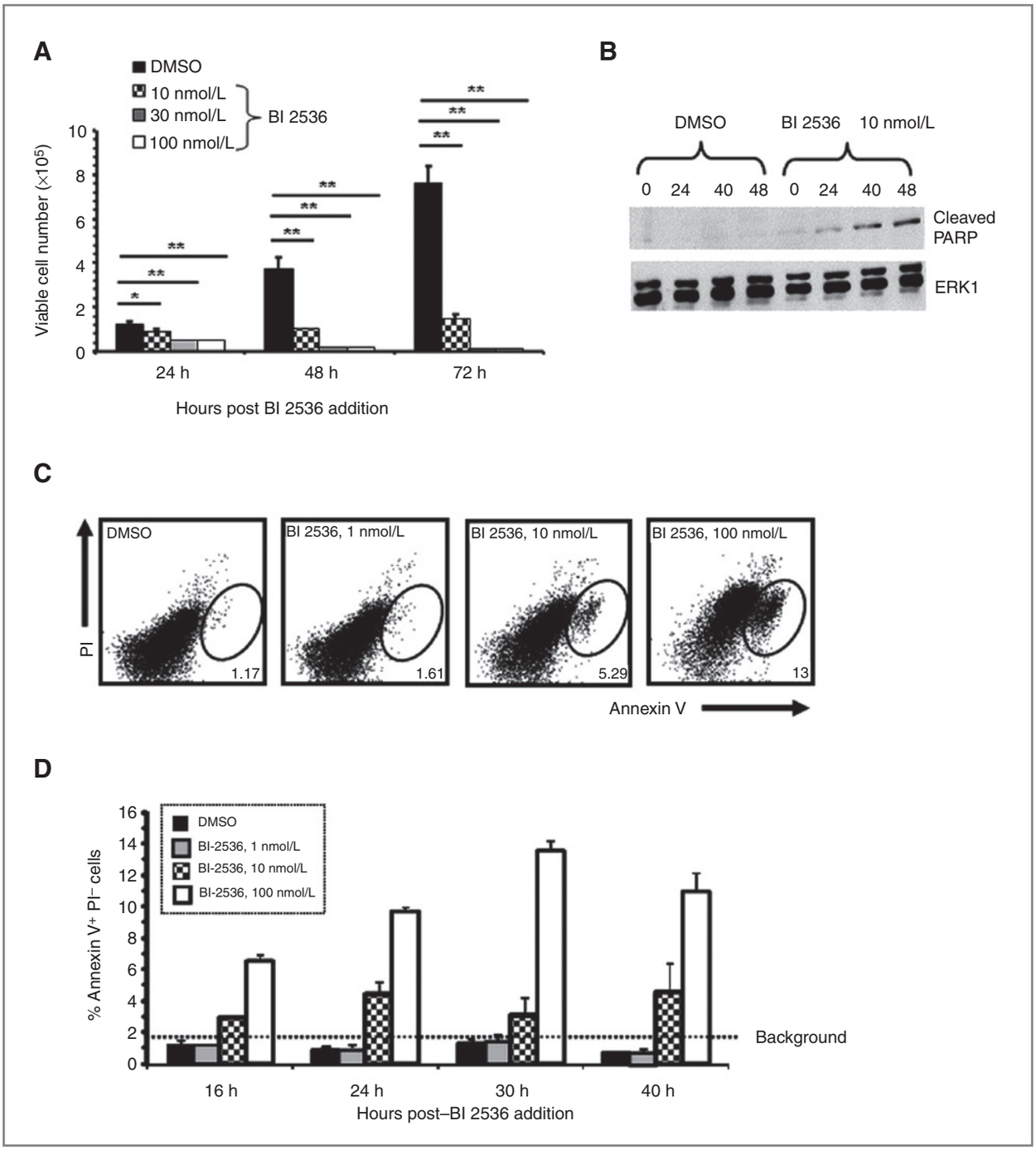

Figure 4. Treatment with BI 2536 induces cell death via apoptosis. A, treatment with BI 2536 (10, 30, and 100 nmol/L) reduces viable cell numbers as assessed by trypan blue exclusion; $n = 3$; *, $P < 0.05$; **, $P < 0.01$. B, representative Western blot demonstrating accumulation of cleaved PARP following treatment with 10 nmol/L BI 2536 ($n = 3$). C and D, accumulation of Annexin V–positive, PI-negative cells following treatment with BI 2563 (1–100 nmol/L). Representative data for 24-hour treatment (C) and quantification of the results at 16 to 40 hours posttreatment (D) are shown ($n = 3$).

BI 2536 suppresses NB tumor growth in a therapeutic xenograft model as a single agent and in combination with irinotecan

We next evaluated the efficacy of BI 2536 treatment on NB tumors *in vivo*. BI 2536 or vehicle was administered intravenously to NOD/SCID mice that had developed NB88R2 xenograft tumors of 50- to 100-mm^3 volume. The mice were first treated with a low drug dose of 12.5 mg/kg given weekly on 2 consecutive days for a total of 3 cycles (Fig. 5A). As shown in Figure 5A, tumor growth

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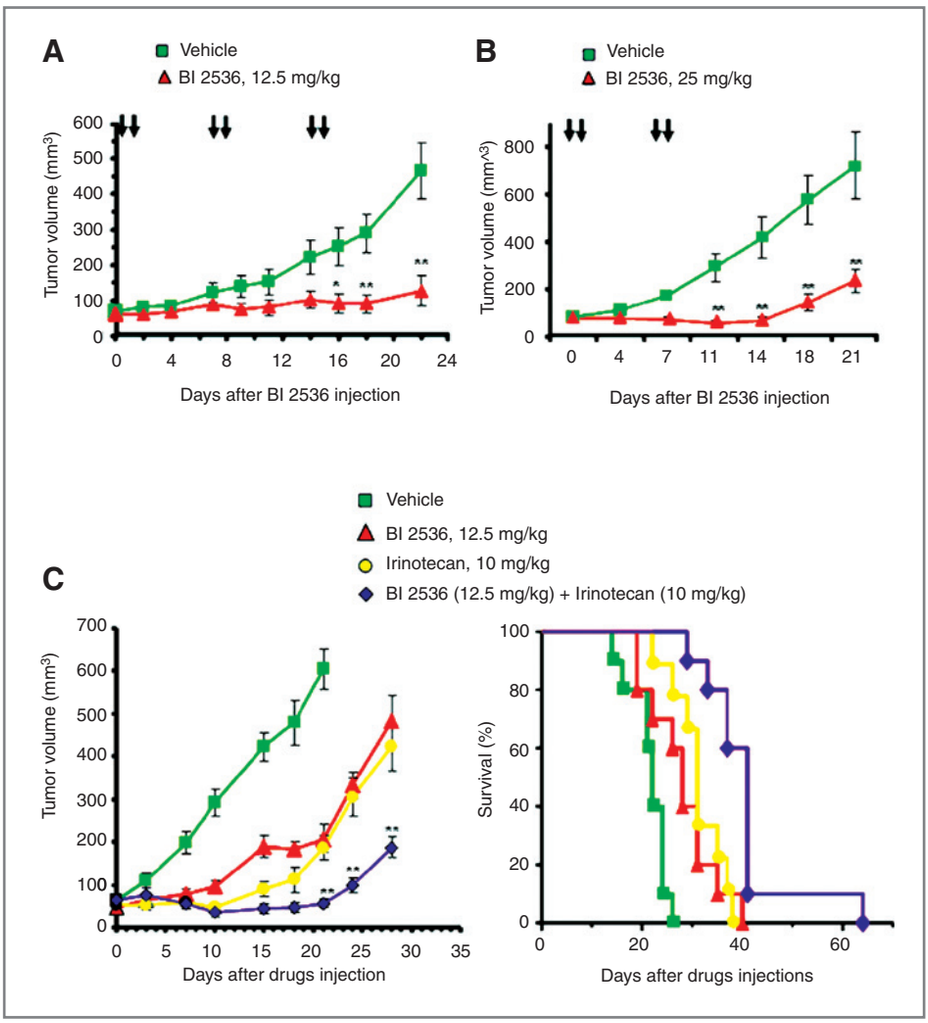

Figure 5. BI 2536 inhibits NB tumor growth *in vivo*. A and B, NOD/SCID mice bearing 50- to 100-mm^3 tumors were injected intravenously with either vehicle (0.1N HCl per saline) or 12.5 to 25 mg/kg BI 2536 for 2 consecutive days a week, for a total of 3 cycles. Two independent experiments were performed in each case with 5 animals per group. Representative tumor growth data are shown; *, $P < 0.05$; **, $P < 0.01$. C, animals with 50- to 100-mm^3 tumors were randomized into 4 groups: group 1 injected intravenously with vehicle (0.1N HCl per saline), group 2 injected intravenously with 12.5 mg/kg of BI 2536 (2 consecutive days, 3 cycles), group 3 injected i.p. with 10 mg/kg of irinotecan (3 doses total, 3 days apart), and group 4 injected with BI 2536 and irinotecan. Both representative tumor growth data and a Kaplan–Meyer survival plot are shown; *, $P < 0.05$; **, $P < 0.01$.

was significantly inhibited following administration of the third cycle of BI 2536 therapy. When mice were administered 25 mg/kg weekly on 2 consecutive days for a total of only 2 cycles, tumor growth was inhibited after the second cycle (Fig. 5B). Toxicity in these animals was limited to a significant reduction in the numbers of neutrophils in the bone marrow (Supplementary Fig. 1), which is the most common side effect described in humans following treatment with BI 2536 (18, 19).

Because new agents for NB will likely be administered in combination with front-line chemotherapeutics, we examined a combination of a low dose of BI 2536 (12.5 mg/kg) with a low dose of irinotecan (10 mg/kg), a topoisomerase inhibitor with demonstrated activity in phase II clinical trials in relapsed NB. Single-agent treatment with low-dose of either BI 2536 or irinotecan was comparably efficacious, resulting in significant growth inhibition, as compared with vehicle-treated animals (Fig. 5C). Treatment with both drugs together resulted in further growth inhibition and improved survival, compared with treatment with the single agents (Fig. 5C). The potency, selectivity, and limited toxicity of BI 2536 in culture and in the xenograft model suggests that this compound and other PLK1 inhibitors merit consideration as novel agents for the treatment of NB.

Discussion

In this study, we screened a kinase inhibitor library to identify candidate protein kinases that play a role in NB TIC survival and self-renewal. To our knowledge, this is the first published report using such libraries in NB and, in particular, in primary human NB TICs from patients with often fatal bone marrow metastases. In this study, we chose to screen NB TICs for a number of reasons. First, NB TICs are primary cells derived from bone marrow metastases of patients with high-risk and often fatal NB that are passaged for a limited number of times in culture (below passage 15). Second, NB TICs express NB and neural crest progenitors markers and have chromosomal aberrations typical of NB tumors (ref. 6); Hansford and colleagues, manuscript submitted). Third, these cells are enriched in tumor-initiating capacity because as few as 10 cells are required to recapitulate

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metastatic NB in immunodeficient mice when injected orthotopically into the adrenal fat pad. Finally, the 3 NB TIC lines used in this study do not carry *MYCN* amplification, which allows dissection of the cellular events in non–*MYCN*-amplified tumor cells that represent approximately 80% of NB patients. Therefore, we believe that NB TICs represent an excellent and relevant model system to study the biology of metastatic NB.

A number of potential kinase targets for NB TIC survival and self-renewal including PI3K/Akt, PKC, Aurora kinase, and Trk were identified. These findings are in agreement with reports showing that Akt or TrkB activity (20, 21) and Aurora A overexpression (22) regulate the proliferation or survival of established NB cell lines from high-risk patients. Moreover, the Trk-selective inhibitor lestaurtinib (CEP-701) has shown potency in a preclinical NB model (23). Our study also revealed several novel promising NB targets, such as ErbB2, PDGFR, PIM1, eEF2K, and CK1 (casein kinase 1), which require further investigation. Two compounds that showed a favorable selectivity profile, JNJ-10198409 (PDGFR) and IC261 (CK1), are currently undergoing further assessment both *in vitro* and *in vivo*.

PLK1 is a serine/threonine kinase that plays an essential role during mitosis (24, 25). Because PLK1 is overexpressed in many cancers and its upregulation often correlates with poor prognosis (12, 24, 26), it has been studied as a potential therapeutic target in adult cancers (11, 13, 16, 27–29). PLK1 has also been suggested to be important in NB cells, as siRNA-mediated PLK1 knockdown induces DNA damage in *MYCN*-amplified, established NB cell lines (30). Our study, using PLK1 siRNA knockdown and 3 PLK1 inhibitors, identifies PLK1 as a critical protein for NB survival, self-renewal, and tumorigenicity. PLK1 inhibitors seem, at least in part, to induce growth arrest and apoptosis in NB TICs by dysregulating and altering the levels of the cell-cycle regulators cyclin B1 and p21. This effect of PLK1 has been shown in a number of cell types (11, 16, 31) in which it promotes cyclin B1 degradation through the phosphorylation of APC (anaphase-promoting complex). Upon PLK1 inhibition or knockdown, the APC-degrading function is impaired, which results in cyclin B1 accumulation and perturbation of the cell cycle. We also observed in BI 2536–treated NB TICs an accumulation of the cyclin-dependent kinase inhibitor p21, a recently identified substrate for PLK1 (17). The dysregulation of these important cell-cycle regulatory proteins may likely induce growth arrest and apoptosis in NB TICs with suppressed PLK1 activity. Taken together, our results identify PLK1 as a required kinase for NB TIC survival and self-renewal and PLK1 inhibitors as candidate NB therapeutic agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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