Supplemental Data

BI 2536, a Potent and Selective Inhibitor of Polo-like Kinase 1, Inhibits Tumor Growth In Vivo

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Supplemental Experimental Procedures

In Vitro Kinase Assays

Recombinant human Plk1 (residues 1–603) was expressed as N-terminal, GST-tagged fusion protein with a baculoviral expression system (BaculoGold, BD Biosciences) and purified by affinity chromatography with Glutathione-agarose (Amersham Biosciences). N-terminal His_6-tagged recombinant human Plk3 (residues 19–301) was purchased from Upstate Cell Signaling Solutions, whereas recombinant human Plk2 was obtained from Invitrogen. Enzyme activity assays for Plk1, Plk2, and Plk3 were performed in the presence of serially diluted inhibitor with 20 ng of recombinant kinase and 10 μg casein from bovine milk (Sigma) as the substrate. Kinase reactions were performed in a final volume of 60 μl for 45 min at 30°C (15 mM $MgCl_2$, 25 mM MOPS [pH 7.0], 1 mM DTT, 1% DMSO, 7.5 μM ATP, 0.3 μCi γ-P^{33}-ATP). Reactions were terminated by the addition of 125 μl of ice-cold 5% TCA. After transfer of the precipitates to MultiScreen mixed ester cellulose filter plates (Millipore), plates were washed with 1% TCA and quantified radiometrically. Dose-response curves were used for calculating IC_{50} values.

Cell-Culture and -Proliferation Analysis

Tumor cell lines were obtained from the American Type Culture Collection or the German National Resource Centre for Biological Material and were cultured according to the supplier's instructions. Cell-proliferation assays were performed by incubation in the presence of various concentrations of BI 2536 for 72 hr, and cell growth was assessed by the measurement of Alamar Blue dye (Serotec) conversion in a fluorescence spectrophotometer. Effective concentrations at which cellular growth was inhibited by 50% (EC_{50}) were extrapolated from the dose-response curve fit.

Cell-Cycle Analysis

Logarithmically growing HeLa cells were incubated with 0.1% DMSO or various concentrations of BI 2536 for 24 hr. For determining the DNA content of the cultures, cell suspensions were fixed in 80% ethanol, treated for 5 min with 0.25% Triton X-100 in PBS, and incubated with 0.1% RNase and 10 μg/ml propidium iodide (PI) in PBS for 20 min at RT. Cell-cycle profiles were determined by flow cytometric analysis (FACSCanto, BD Biosciences).

Immunofluorescence Microscopy

Cells were grown on coverslips and fixed with 4% formaldehyde in PBS at RT or −20°C methanol for 15 min. After fixation, samples were permeabilized with 0.5% Triton X-100 in PBS for 15 min and thereafter blocked with 10% FCS in PBS containing 0.01% Triton X-100. Coverslips were incubated for 1 hr at room temperature with primary and secondary antibodies and mounted with ProLong Gold (Molecular Probes) onto slides. Images were taken on a Zeiss Axioplan 2 microscope with 63× or 100× Plan-Apochromat objective lenses (Carl Zeiss, Jena) and a CoolSnapHQ CCD camera (Photometrics).

Antibodies were used at the following dilutions: mouse anti-α-tubulin (clone B-5-1-2, Sigma) 1:5000; rabbit anti-γ-tubulin (T3559, Sigma) 1:4000; rabbit anti-Mad2 (Covance) 1:500; PARP p85 fragment (9541, Upstate). Alexa 488-, Alexa 568-, and Alexa 633-labeled secondary antibodies as well as DAPI were from Molecular Probes (Invitrogen). Western blotting was performed as described in [S1].

Animal Efficacy Studies

Female BomTac:NMRI-Foxn1^{nu} mice (Bomholtgard/Taconic) were grafted subcutaneously with HCT 116 colon-carcinoma, NCI-H460, or A549 lung-carcinoma cells by subcutaneous injection, respectively, of 2×10^6, 1×10^6, and 1×10^7 cells into the flank of each mouse. When tumors reached a volume of approximately 50 mm^3, animals were pair-matched into treatment and control groups of ten mice each. In regression experiments, treatment was not initiated until the mean tumor volume reached 500 mm^3. BI 2536 was formulated in hydrochloric acid (0.1 N), diluted with 0.9% NaCl, and injected intravenously into the tail vein at the indicated dose and schedule. The administration volume was 10 ml per kg body weight. Tumor volumes were determined three times a week with a caliper. The results were converted to tumor volume (mm^3) by the following formula: length × width^2 × π/6. The weight of the mice was determined as an indicator of tolerability on the same days. For statistical analysis, the treatment group was compared with the vehicle control group in a one-sided (decreasing) exact Wilcoxon test.

Immunohistochemistry

For the analysis of histone H3 phosphorylation and apoptosis in tumor sections, female BomTac:NMRI-Foxn1^{nu} mice were grafted subcutaneously with NCI-H460 lung-carcinoma cells as described above. Mean tumor volume at the start of the experiment was approximately 100 mm^3. NCI-H460 lung tumors were excised from nude mice before treatment, 24 and 48 hr after i.v. administration of 60 mg/kg BI 2536. Histone H3 phosphorylation was determined by the avidin-biotin immunoperoxidase (ABC) method on frozen sections with an antibody to Ser-10-phosphorylated histone H3 (16-189, Upstate) and followed by hematoxylin counterstaining. Apoptosis was quantified with a TUNEL assay, with the ApopTag Fluorescein In Situ Apoptosis Detection Kit (S7160, Chemicon International) and analyzed by fluorescence microscopy.

Near-Infrared Fluorescence Imaging

For the analysis of the induction of apoptosis by near-infrared fluorescence imaging, female BomTac:NMRI-Foxn1^{nu} mice were grafted subcutaneously with NCI-H460 lung-carcinoma cells in the chest wall as described above. When tumors reached a size of approximately 100 mm^3, animals were treated i.v. with BI 2536 at a dose of 60 mg/kg or with the control vehicle only; n = 4 animals per group. Mice received the imaging probe (Cy-Annexin V) by tail-vein injection (75 μg/mouse) 47 hr after BI 2536 treatment, and anesthetized animals (isoflurane) were imaged 60 min later with the AEQUORIA reflectance imaging system (Hamamatsu Photonics). Optical images were digitally acquired by means of a highly sensitive CCD camera (ORCA II-BT-512) as visible light image (to outline the animal) and as fluorescent image from the skin surface (exposure time 0.5 s) in the NIRF channel. SimplePCI Software 6.1 (Compix) was used for image acquisition, processing, and data analysis.

Magnetic Resonance Imaging

For the measurement of the apparent diffusion coefficient of water (ADC_w), female BomTac:NMRI-Foxn1^{nu} mice were grafted subcutaneously with HCT 116 colon-carcinoma cells as described above. When tumors reached a size of approximately 50 mm^3, two doses of 50 mg/kg of BI 2536 were administered i.v. on two consecutive days (n = 6 animals). A separate group of animals (n = 6) with same-size tumors was vehicle treated and served as a control. MRI measurements were performed on the PharmaScan 70/16 MR system (Bruker Biospin) before the treatment and 2, 4, and 7 days

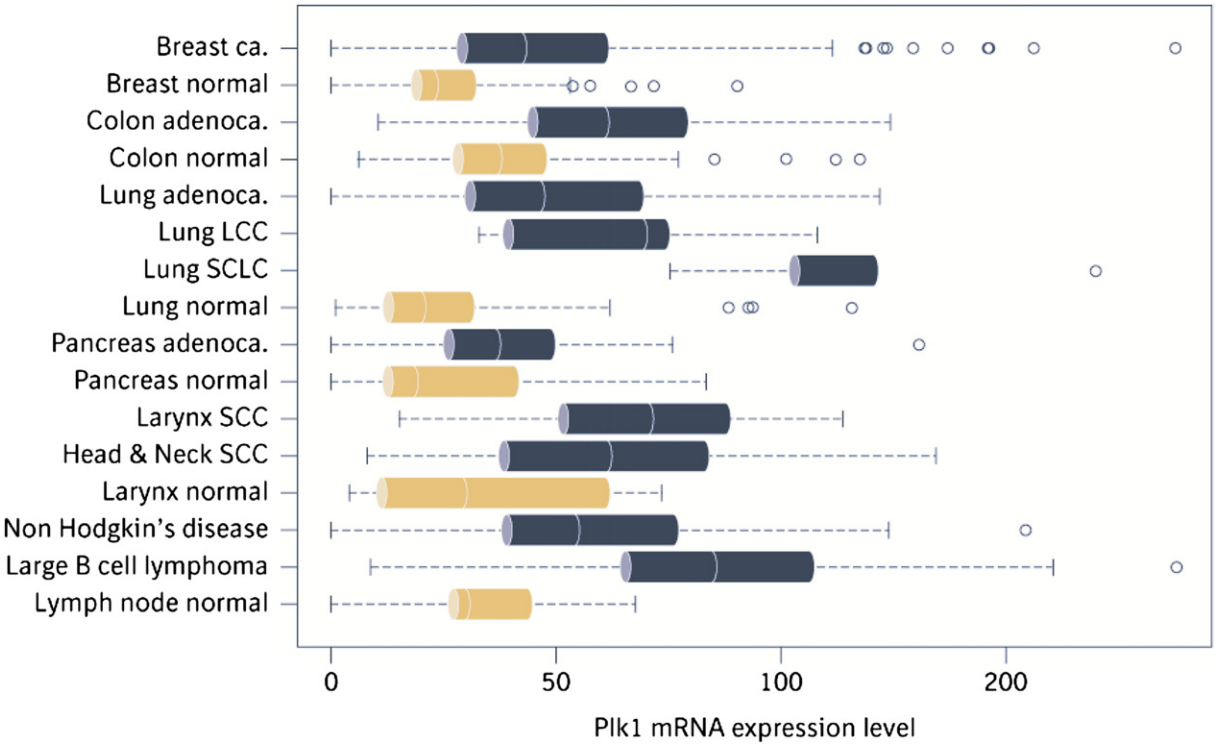

Figure S1. mRNA Expression Profile of Plk1 in Normal and Malignant Human Tissues

The panel shows whisker-box plots for normal tissues (brown) and malignant lesions (blue). The vertical lines in each box indicate median values, and box limits represent the first and third quartiles; the whiskers extend to 1.5 times this interquartile range. The circles represent outliers. Abbreviations used are as follows: LCC, large-cell carcinoma; SCLC, small-cell lung carcinoma; SCC, squamous-cell carcinoma. The number of samples for each tissue from top to bottom is n = 278, 84, 95, 215, 73, 10, 5, 134, 66, 51, 11, 27, 6, 175, 64, and 14, respectively. The whisker-box plots were generated with the eNorthern tool of the GeneExpress Genesis 2.5 software, which is based on normalized gene-expression data extracted from the BioExpress database (Gene Logic) as reported [S2]. The hybridizations were performed on Affymetrix HG-U133A/B oligonucleotide arrays.

after initiation of the treatment. Animals were anesthetized, body temperature was kept constant, and breath frequency was monitored during the duration of the measurement. ADC_w were measured with a manufacturer-supplied software package (ParaVision 3.0.2, Bruker Biospin) in a 2-mm-thick single axial slice comprising subcutaneous tumor, spinal muscle, and the abdomen of the animals.

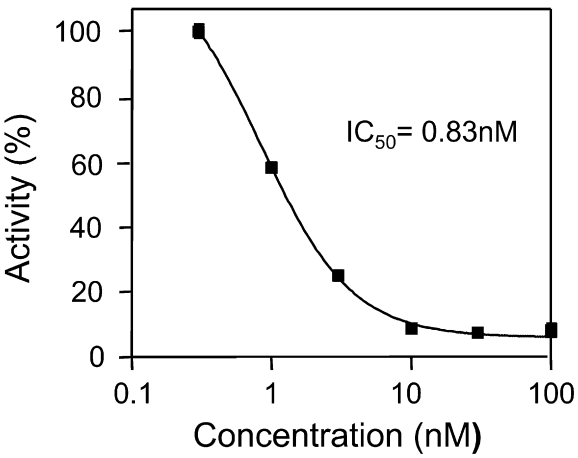

Figure S2. BI 2536 Is a Potent Plk1 Inhibitor

The figure shows the activity of BI2536 in a Plk1 in vitro kinase assay at the indicated concentrations of the compound (n = 8 per concentration; data are mean ± SEM). For assay details, see Experimental Procedures.

Supplemental References

S1. Hauf, S., Roitinger, E., Koch, B., Dittrich, C.M., Mechtler, K., and Peters, J.M. (2005). Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. PLoS Biol. *3*, e69.

S2. Shen-Ong, G.L., Feng, Y., and Troyer, D.A. (2003). Expression profiling identifies a novel alpha-methylacyl-CoA racemase exon with fumarate hydratase homology. Cancer Res. *63*, 3296–3301.

S3. Gumireddy, K., Reddy, M.V., Cosenza, S.C., Boominathan, R., Baker, S.J., Papathi, N., Jiang, J., Holland, J., and Reddy, E.P. (2005). ON01910, a non-ATP-competitive small molecule inhibitor of Plk1, is a potent anticancer agent. Cancer Cell *7*, 275–286.

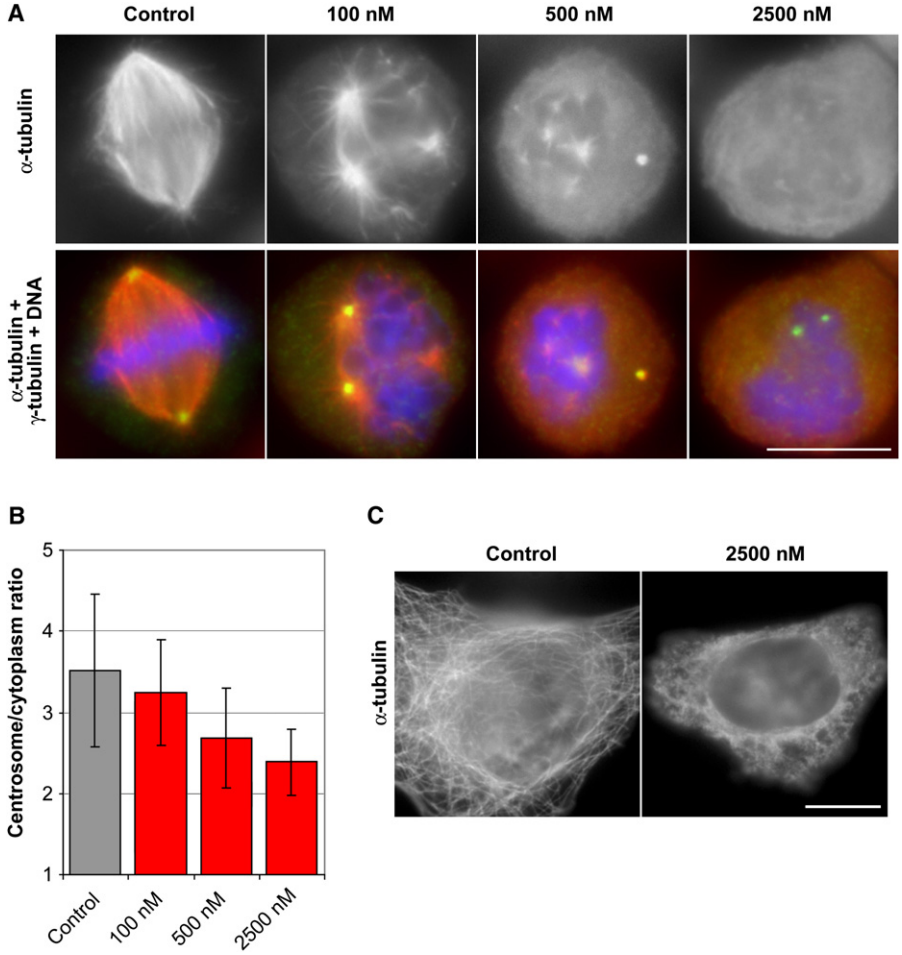

Figure S3. Phenotypes Caused by ON01910 Are Distinct from BI 2536 and Plk1 RNAi

(A) Asynchronous populations of HeLa cells were treated for 2 hr with the indicated doses of ON01910, fixed, and processed for immunofluorescence with antibodies against α- and γ-tubulin. Prominent phenotypes for each of the concentrations are shown. γ-tubulin is shown in green; α-tubulin is shown in red; DAPI is shown in blue. ON01910 was synthesized according to the structural information provided by Gumireddy et al. [S3]. The scale bar represents 10 μm.

(B) Quantification of centrosomal γ-tubulin signals in (A), n = 20 each. Data are mean ± SEM.

(C) α-tubulin immunostaining of cells treated for 2 hr with or without 2.5 μM ON01910. The scale bar represents 10 μm.

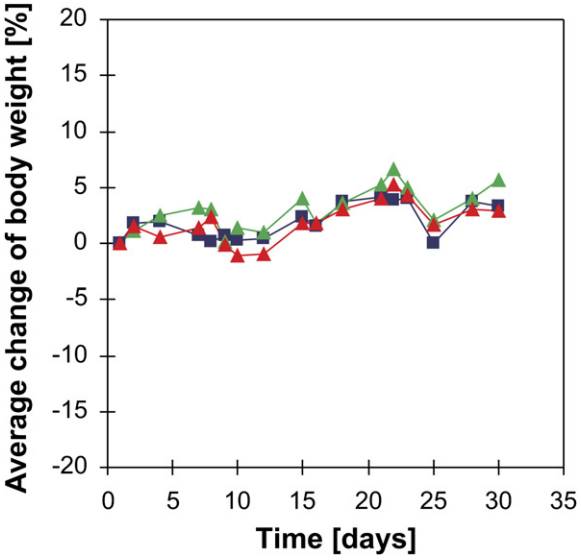

Figure S4. Body-Weight Change of Mice Treated with Vehicle Control or BI 2536

Nude mice bearing established HCT 116 tumors were treated i.v. for four cycles with the vehicle control (indicated by blue squares), BI 2536 at a dose of 40 mg/kg once weekly (indicated by green triangles), or twice weekly on two consecutive days (indicated by red triangles); n = 10 animals per group. Average change of body weight in percentage is shown.

Table S1. Kinase-Selectivity Profile of BI 2536

Kinase	IC50 [nM]	Kinase	IC50 [nM]
Plk1	0.83	JNK1	>10,000
Plk2	3.5	Lck	>10,001
Plk3	9.0	Lyn	>10,000
Abl	>10,000	MAPKAP-K2	>10,000
Axl	>10,000	MEK1	>10,000
AMPK	>10,000	Met	4,754
Aurora A	>10,000	MSK1	>10,000
Aurora B	>10,000	MKK1	>10,000
ßIRK	>10,000	Nek6	>10,000
B-RAF	>10,000	p38α	>10,000
Btk	>10,000	p38β	>10,000
CDK1/B1	>10,000	p38γ	>10,000
CDK2/E	>10,000	p38δ	>10,000
CHK1	>10,000	PAK2	>10,000
CK1	>10,000	PDGFRα	>10,000
CK2	>10,000	PDGFRβ	>10,000
C-RAF	>10,000	PDK1	>10,000
CSK	>10,000	PI3Kα	2,407
DYRK1A	>10,000	PKA	>10,000
ECK	>10,000	PKBα	>10,000
ErbB4	9,908	PKBβ	>10,000
ERK2	>10,000	PKCα	>10,000
FGFR1	>10,000	PRAK	>10,000
FGFR3	>10,000	Ret	>10,000
Flt1	>10,000	ROCK2	>10,000
Flt3	>10,000	Ron	>10,000
GSK3ß	>10,000	S6K	>10,000
Hek	>10,000	SGK	>10,000
HER2	>10,000	Src	>10,000
HGFR	9,500	Syk	>10,000
ITK	>10,000	Tie2	6,549
JAK2	>10,000	VEGFR1	>10,000
JAK3	>10,000	VEGFR3	>10,000

We assessed the inhibitory activity of BI 2536 against a panel of tyrosine and serine/threonine kinases. Kinase assays were performed by contract research organizations, or reagents were purchased from commercial sources and assays were performed according to the supplier's protocols. Appropriate positive and negative controls were included in the assay design.