Supplemental material

Patients and tumors

Luminal A, luminal, TNBC and HER2 biopsies were obtained from patients treated at Curie hospital. Normal breast tissues were obtained from reduction mammoplasty. Samples (DNA, RNA, biopsies) were provided by the Biological Resource Center of Curie Institute. Tumors contained between 50-90% tumor cells, as revealed by haematoxylin-eosin-safran (HES) staining. IHC was performed as described (1). Positive nuclear staining for estrogen (ER) and progesterone (PR) receptors were recorded in accordance with standardized guidelines, using 10% as the cut-off for ER and PR positive cells. For HER2, only staining of membranes was considered with a 30% cut-off as recommended. The tumors of our cohort have been characterized by IHC as follows: luminal A (ER+, PR+, HER2-), luminal B (ER+, PR+, HER2+/−), HER2 (ER-, PR-, HER2+), TNBC (ER-, PR-, HER2-). The grades of the tumors were as follow: luminal A (34 grade I, 1 grade II), luminal B (2 grade II, 38 grade III), HER2 (1 grade II, 32 grade III), TNBC (46 grade III).

For PLK1 staining, tissue microarrays (TMA) containing alcohol, formalin and acetic acid (AFA)-fixed paraffin-embedded tissues were made as described (1). Antigen retrieval was performed in EDTA buffer pH=8 (Lab Vision) for 20 min at 95°C. Endogenous biotins were blocked by Biotin blocking system (Dako Cytomation). After washes in Phosphate Buffer Saline (PBS)-Tween buffer, endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 5 min then rinsed in distilled water. Each tissue section was blocked with a solution of PBS (pH 7.4) containing 1% of BSA and 1.4% of normal donkey serum for 5 min, followed by one hour incubation at room temperature with primary antibody against PLK1. After washes, slides were incubated with rabbit biotinylated antibody (Jackson Immunoresearch) for 30 min. Immunostaining was revealed using the Vectastain ABC
peroxidase system (Vector Laboratories) with diaminobenzidine (DAB) as a chromogen. Slides were counter-stained with haematoxylin before mounting. The reactions were carried out using an automated stainer (LabVision). Omission of the primary antibody was used as a negative control. IHC conditions were first optimized using cell pellets from cell lines expressing high or low levels of PLK1. To assess whether the mean percentage of stained cells differs between any two subtypes, we performed a two-sample Wilcoxon test. Overall we made 6 tests and account for multiple-testing issue using the Bonferonni correction.

ER (clone 6F11/2, 1/50 dilution, Novocastra), PR (clone 1A6, 1/200 dilution, Novocastra), HER2 (clone CB11, 1/800 dilution, Novocastra) and PLK1 (clone 208G4, dilution 1/50, Cell Signaling Technology) antibodies were used.

**Clinical data**

Disease-free interval was defined as the time from the diagnosis of breast cancer to the occurrence of a locoregional, distant or controlateral recurrence. Patients with initial metastatic disease or with a previous aggressive cancer were excluded. The number of events was insufficient to perform the analysis in luminal A and B subgroups. TNBC (n=39) tumors were analyzed. Kaplan–Meier survival plots and log-rank tests were used to assess the differences in survival curves.

**Western-blotting and Reverse Phase Protein Array (RPPA)**

Western-blotting was performed as previously described (1). PLK1 expression was studied using RPPA technology in the different tumor subtypes: TNBC (n=44), HER2+/ER- (n=28), luminal A (n=24) and luminal B tumors (n=38). RPPA is a miniaturized dot-blot technology based on robotic printing of a large number of different cell/tissue lysates onto nitrocellulose bound to histology slides. It allows the printing of very small quantities of protein (about 1ng per spot), convenient for the analysis of minimal quantities of biopsy material. Briefly, the
proteins were extracted from frozen tumor samples and then processed according to the protocol previously detailed (1) with some modifications. Samples were printed using an Aushon 2470 printer (Aushon Biosystems, Billerica, MA, USA). Tumor samples were deposited in single concentrations, while control cell lines and pools of tumor samples were deposited as serial dilutions in order to obtain the antibody response curve. RPPA was performed using an Autostainer (DAKO, Glostrup, Denmark) according to the protocol previously described (1), except that no double staining with actin was performed, that the washes after signal amplification did not contain DMSO and that a streptavidin-Alexa648 was used for the fluorescent labeling. PLK1 was detected with a highly specific antibody that has been first validated for RPPA use in various cell lines by Western-blotting (data not shown). The RPPA data for PLK1 was part of a larger set of 130 RPPA arrays that were used to analyze the same tumor samples with different antibodies. Briefly, the data from each RPPA slide was log2 transformed and scaled, a linear model was used to correct for effects from an experimental batch, and sample loadings were adjusted individually for each RPPA by correcting the dependency of the data for individual arrays on the median value of each sample over all 130 arrays using a linear regression.

PLK1 (clone 208G4, 1/1000 dilution), cleaved-PARP (clone 19F4, 1/2000 dilution), cleaved-caspase 7 (1/1000 dilution), Histone H2AX (1/2000 dilution), cleaved-caspase 8 (clone 18C8, 1/1000 dilution) antibodies were from Cell Signaling Technology; cleaved-caspase 3 (clone E83-77, 1/500 dilution) antibody was from Epitomics; phospho-histone H2AX (Ser139) (clone JBW301, 1/2000 dilution) antibody was from Upstate-Millipore; actin (clone AC-15, 1/5000 dilution) antibody was from Sigma-Aldrich.

Microarray data preprocessing of tumors and cell lines

DNA, RNA and protein lysates were purified as described (1).
For the DNA copy number, the *PLK1-PLK4* genes were analyzed in the different tumor subtypes: TNBC (n=46), HER2+/ER- (n=33), luminal A (n=35) and luminal B (n=39), and in healthy tissue samples (n=17). DNA was extracted from frozen tumor samples with the use of a standard phenol/chloroform procedure. Genomic DNA (500 ng) were used to hybridize Affymetrix SNP6.0 microarrays, according the manufacturer recommendations. The data were first normalized using Affymetrix Genotyping Console. Next, the signal from SNP probes was segmented using the colibri function available in the CGHseg R package (2) to identify copy number alterations, and a first segmentation round was used to identify and discard outlier probes giving rise to singleton segments. After a second segmentation round, segments that contained less than five probes were merged to the closest adjacent segment. For every sample, a smoothed signal was produced by setting the values of all probes within one segment to the average of probes on that segment. We defined the thresholds for gain and loss as the .999 and .001 quantiles of the distribution of smoothed probe signal levels from 17 healthy breast tissue samples.

*PLK1-PLK4* mRNA levels were analyzed in the different tumor subtypes: TNBC (n=41), HER2+/ER- (n=30), luminal A (n=29) and luminal B (n=30), and in healthy tissue samples (n=11). Total RNA was extracted from frozen tumor samples with the RNeasy Mini Kit (Qiagen, Courtaboeuf, France), then the RNA clean up kit (Macherey Nagel, Hoerdt, France), in accordance with the manufacturer's instructions. After RNA quality and quantity controls, 2µg of total RNA were amplified and labeled according to the Affymetrix one cycle synthesis target preparation protocol to hybridize Affymetrix U133 plus 2.0 chips. The data were analyzed using the brainarray HGU133Plus2_Hs_ENTREZG version 13 custom chipset definition file (3). The data were first normalized using GC-RMA (version 2.14.1) (4). A linear mixed model was then fitted using the nlme package using Restricted Maximum
Likelihood (REML) (5). The model included the different experimental batches and the sample type as fixed effects, and treated technical variations as random effects. No interaction terms were included. This model was used to derive significance for differential expression between different tumor types and for correction of batch and hybridization effects. For further analysis, technical replicates were averaged. From the distribution of the GC-RMA normalized data, a clear bi-modal distribution of intensities can be observed, which can be interpreted as the presence of a large number of probes with noise-level signal. We chose to discard such probesets from this analysis: specifically, we considered as not expressed, and therefore discarded, all the probesets with a log2 intensity of less than 4 in 95% or more of the tumor and normal tissue samples. This filter reduced the number of probesets from 18123 to 11543, each uniquely matching an Entrez gene entry.

Cell culture
MDA-MB-231 cells are from Mina Bissell’s laboratory (Berkeley, California, USA). HCC38, HCC70 and HCC1937 were maintained in RPMI-1640 with 10% fetal calf serum (FCS) (Invitrogen, Cergy Pontoise, France), 1.5 g/l sodium bicarbonate (Invitrogen), 10 mM Heps (Invitrogen) and 1 mM sodium pyruvate (Invitrogen). BT20 were cultured in MEM(Eagle) containing 10% FBS, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino-acids, and 1 mM sodium pyruvate. MDA-MB-468 cells were grown with RPMI with 10% FCS. MCF10A were cultured in DMEM-F12 containing 5% horse serum, 20ng/ml EGF, 100ng/ml cholera toxin, 0.01mg/ml human insulin and 500ng/ml hydrocortisone. MDA-MB-231 were cultured in DMEM-F12 containing 10% FCS. All the cells were cultured in the presence of 100U/mL penicillin and 100µg/ml streptomycin (Invitrogen). Lysates were prepared at 60-90% cell confluency and analyzed by Western-blotting.

RNA interference
Cells were seeded in 96-well plates, in triplicate or quadruplicate wells at a density of 6,000 cells per well for MDA-MB-468, HCC70, BT20; 4,000 cells per well for HCC1937; 3,000 cells per well for MCF10A and MDA-MB-231. When 6-well plates were used, MDA-MB-468 and MCF10A were seeded at 250,000 cells per well and 100,000 cells, respectively. Twenty four hours later, MCF10A, MDA-MB-468 and MDA-MB-231 were transfected using Lipofectamine RNAiMax according to the manufacturer’s instructions (Invitrogen) with 20 nM of either a control RNAi (AllStars) or 2 distinct PLK1 RNAi (PLK1#6, PLK1#7) (all from Qiagen). BT20 and HCC1937 were transfected using Lipofectemine 2000 according to the manufacturer’s instructions (Invitrogen). HCC70 were plated and transfected at the same time. Twenty-four, 48 and 72 hours post-transfection, cells were analyzed directly in plates or collected for further studies.

Cell proliferation assay

Cells were seeded into 96-well plates at a density determined on the basis of the growth characteristics of each cell line (1500 cells/well for MDA-MB-468, BT-20, HCC1937, HCC70; 750 cells / well for MDAMB231; 375 cells/well for MCF10A). Forty eight hours later, cells (triplicate wells) were treated for four doubling time (i.e., 7 days for MDA-MB-468, BT-20, HCC1937, HCC70; 4 days for MDAMB231 and 3 days for MCF10A) with various concentrations (0.2-50 nM) of a PLK1 inhibitor (BI-2536) (Selleck Chemicals) or DMSO (Sigma-Aldrich) as a control. When PLK1 was depleted, cells were subjected to the cell proliferation assay 24, 48 or 72 hours post-transfection with RNAi. The metabolically active cells were determined on the basis of mitochondrial conversion of 3-(4,5-diethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT assay) to formazin. Fifteen µL of MTT (5 mg/ml in PBS) was added to each well. After 4h of incubation at 37°C, floating plus adherent cells were lysed by the addition of 10 % SDS in 10 mM HCl. The absorbance
was measured at the wavelength of 540 nm (Infinite 200, Tecan) and results are presented as the percentage of control cell growth inhibition obtained from untreated cells grown in the same culture plate. The IC50s were determined on the basis of the dose-response curves.

**Caspase 3/7 assay**

Cells were harvested and seeded in 96-well plates (6,000 cells/well for MDA-MB-468 and 3,000 cells/well for MCF10A). After overnight growth, cells were treated in triplicate with various concentrations of BI-2536 or DMSO as a control. Twenty-four, 48 and 72 hours later, caspase activity was determined by using Caspase-Glo® 3/7 luminescent assay (Promega, Charbonnières-les-Bains, France), according to the manufacturer’s instructions. Results are presented as caspase 3/7 activity normalized by caspase 3/7 activity from vehicle-treated cells or control RNAi-transfected cells.

**Cell cycle analysis**

MDA-MB-468 and MCF10A were transfected with RNAi. Twenty four and 48 hours post-transfection, they were collected by trypsinisation, washed once with PBS, then with PBS containing 0.5% BSA and fixed in cold 70% ethanol. After fixation, cells were washed with PBS containing 0.5% BSA and incubated with PBS containing 200 µg/ml RNase A (Invitrogen) and 10 µg/ml propidium iodide (Invitrogen) for 30 min at room temperature. Flow cytometry was carried out with FACScalibur (Becton Dickinson, Le Pont de Claix, France) using Cellquest software (Becton Dickinson) to determine cellular DNA content. A minimum of 20,000 events were counted. DNA content was analyzed and quantified using Modfit LT software (Verity Software House). Results are expressed as percentage of cells in each of the cell-cycle phases plus the sub-G1 population representing apoptotic cells.
Annexin V assay

After transfection with PLK1 and control RNAi, MDA-MB-468 and MCF10A cells were harvested 48h later, stained for annexin-V-FLUOS / propidium iodide (PI), using the annexin-V-FLUOS Staining Kit (Roche), then analyzed on LSRII Instrument (Becton Dickinson) using Cellquest software (Becton Dickinson). A minimum of 10,000 events were counted. Relative numbers of viable (annexin V-negative / PI-negative), necrotic (PI-positive / annexin V-negative) and apoptotic (annexin V-positive) cells were determined.

Soft-agar tumorigenicity assay.

A 1 ml bottom layer consisting of 0.5 % agar medium (equal volumes of 1% agar and 2× culture medium) was added to six-well plates. MDA-MB-468 and HCC70 cells were transfected with RNAi, and 24h later, they were trypsinized, resuspended in 0.35% agar medium, and plated at 5000 cells / well as a top layer. Cells were incubated 4 weeks at 37°C and the colonies were stained with a MTT assay. Plates were photographed with Fujifilm LAS-3000 Imager, and the clones were quantified using Image J software.

Three-dimensional cell culture

Three-dimensional cell culture was performed with Matrigel (BD Biosciences) as previously described (6). MDA-MB-468 cells were grown in the 3D on-top assay for 7 days to form grape-like 3D structures and MCF10A for 10 days to form round 3D structures. After 7 or 10 days, cells were treated with various concentrations of BI-2536 (or DMSO used as a control). Three days later, cells were analyzed directly in plate or collected for further studies. Cell viability, performed on 24-well plates, was determined using the Cell Proliferation Assay Kit (Millipore): 100 µl of WST1 solution was added to each well; after 2h incubation at 37°C, the absorbance was measured at the wavelength of 450 nm (Infinite 200®, Tecan, Lyon, France)
and results are presented as the percentage of growth of treated cell compared to control cells. Seventy two hours after treatment, MDA-MB-468 or MCF10A were isolated from the Matrigel, as previously described (6), and cells were lysed, as described (1) for analysis by western-blot.

**Mice, compounds, treatment and tumor growth measurement**

Female Swiss nude mice, 5-7 week old, were purchased from Charles River (Les Arbresles, France) and maintained in specific pathogen-free conditions. Their care and housing were in accordance with institutional guidelines as put forth by the French Ethical Committee. Doxorubicin and cyclophosphamide (Endoxan) were purchased from Teva Pharmaceuticals (Paris, France) and Baxter (Maurepas, France), respectively. The doxorubicin and cyclophosphamide combination was designated as DC. Human TNBC breast cancer xenograft models (HBCx-10 and HBCx-24) were established as detailed elsewhere (7-9). HBCx-10 represents a xenograft model of DC-induced complete remission and tumor recurrence (7, 8) whereas HBCx-24 does not respond to DC (data not shown). Briefly, tumor fragments (30-60 mm3) were grafted into the inter-scapular fat pad of nude mice. When tumors reached a size of 60-200 mm3, the mice were randomly assigned to the control or treatment groups. Treatments were started on day 1. The PLK1 inhibitor, BI-2536 (Selleck Chemicals), was formulated at 5 mg/ml in a mixture of 10% ethanol + 3% polysorbate 80 + 2% hydroxypropyl beta cyclodextrine 10% and then diluted in 0.9% NaCl at a working concentration of 2 mg/ml. BI-2536 toxicity studies was performed with administration of 10, 20 and 50 mg/kg, twice a week, intraperitoneally (IP). Treatments with 10 and 20 mg/kg were not associated with any mortality and body weight loss (data not shown). Mice bearing human breast cancer xenografts were treated with BI-2536 (IP) alone at a dose of 20 mg/kg, twice a week, or combined with doxorubicin (2 mg/kg, IP) and cyclophosphamide (100 mg/kg, IP) at days 1,
Tumor growth was evaluated by measuring two perpendicular tumor diameters with a caliper, twice a week. Each tumor volume (V) was calculated according to the following formula: V = a × b²/2, where a and b are the largest and smallest perpendicular tumor diameters. Relative tumor volumes (RTV) were calculated from the following formula: RTV = (Vₓ/V₁), where Vₓ is the tumor volume on day x and V₁ is the tumor volume at initiation of therapy (day 1). Antitumor activity was evaluated according to tumor growth inhibition (TGI), calculated according to the following formula: percent of TGI = 100 - (RTVₜ/RTVₖ × 100), where RTVₜ is the median RTV of treated mice and RTVₖ is the median RTV of controls, both at a given time point when the antitumor effect was optimal. Growth curves were obtained by plotting the mean values of RTV on the Y axis against time (X axis, expressed as days after start of treatment). Mice were ethically killed when the tumor volume reached 2,000 mm³.

References